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Humic Substances in the Aquatic and Terrestrial Environment

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Preface

In 1986 a Nordic conference on humic substances was arranged in Oslo, Norway. The conference was very successful, and it was suggested that there should be continued regular meetings for Scandinavian scientists working within this area. Linköping University accepted the responsibility of hosting the second meeting in this series.

At an early stage it was decided that the meeting should also be open to non-Nordic participants. Thus, the International Symposium on Humic Substances in the Aquatic and Terrestrial Environment, August 21-23, 1989, in Linköping, Sweden, attracted about 120 participants from 19 nations. A total of 71 contributions were presented, orally or as posters. Papers from both the oral and poster sessions have been collected in this proceedings volume, in which the chapters have the same titles as the oral sessions of the symposium. Each chapter (with the exception of Chap. 2) starts with a paper based on the plenary lecture of the session.

The program and final selection of papers were made by the Scientific Committee (Prof Bert Allard, Linköping University; Dr Göran Bengtsson, University of Lund, Sweden; Dr Hans Borén, Linköping University; Dr James Ephraim, Linköping University; Dr Egil Gjessing, National Institute of Public Health, Norway; Prof Anders Grimvall, Linköping University; Prof Bjarne Holmbom, University of Åbo Academy, Finland; Dr Ronald Malcolm, US Geological Survey, USA; and Prof Robert Petersen, University of Lund, Sweden). The assistance of Ms Irina Arsenie, Ms Gunilla Asplund, Ms Maria Nordén and Ms Catharina Pettersson, and also of the members of the Scientific Committee, in editing and preparing the conference proceedings is gratefully acknowledged, as well as the excellent help with word processing and lay-out by Ms Susanne Eriksson, Ms Lisbeth Thornbury and Ms Pia Sandholm.

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Bert Allard, Hans Borén and Anders Grimvall

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Introduction

The Different Roles of Humic Substances in the Environment

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Humic substances comprise a class of biogenic, coloured, organic substances that are ubiquitous in soil, sediment and water. Originally, the occurrence and nature of humic substances were regarded as issues of primarily academic interest. This situation is now rapidly changing, and studies of humics have gained recognition as important contributions to environmental science. In particular it has been shown that humic substances, in several different ways can interact with biologically active substances, thereby modifying their environmental impact (see Table 1).

Whereas the history of soil humus studies goes back to the 19th century, the awareness of aquatic humus is more recent. The brownish colour that, in many surface waters, shows the presence of substantial amounts of humic substances, was long considered to be a harmless phenomenon that did not call for detailed investigations. Humic waters had few known toxic effects, and the refractory character of humic substances indicated the they played a peripheral role in most biochemical processes. In fact, it was not until the mid 70's that aquatic humus was brought into focus in environmental science. The event trigging this was the discovery of the interaction between humic substances and chlorine used for disinfection of drinking water. Toxic substances, such as chloroform, were detected in all chlorinated waters, and humic substances were identified as the main precursors.

The role of humics in the mobilization and subsequent transport of trace elements in the environment was recognized for the first time in the early 80's. This role was considered to be of particular importance in connection with geologic storage of high-level radioactive waste. In water with "normal" concentration levels of humic compounds, the speciation of e.g. the trivalent actinides, would be entirely dominated by the complexation with these agents.

Table 1 Some different roles of humic substances in the environment.

Complexing agent or adsorbent for man-made pollutants	Complexing agent or adsorbent for naturally occurring substances	Precursor of toxic substances in technical processes	Precursor of toxic substances in natural processes

The topics of this conference (Session 1 - Isolation, fractionation and characterization; Session 2 - Biological and chemical transformation and degradation; Session 3 - Complex formation and interactions with solids; Session 4 - Biological activity; and session 5 - Halogenation of humic substances) were selected to represent areas of current environmental interest.

As described by Malcolm (Chapter 1), developement of isolation procedures, as well as fractionation methods and characterization schemes, are still topics of considerable interest. Isolation procedures are being refined and information about elemental composition, functional groups and molecular weight of natural organics is accumulating. The tendency in the obtained results is clear: even if isolates of humic substances are purified by the best techniques available, the resulting mixtures of organics are very complex. In addition, it is obvious that humics of different origin can differ significantly with respect to elemental composition, molecular weight, etc. This implies that one cannot expect to find a simple structural characterization of humic substances. Another implication is that further studies of the humics are likely to reveal new aspects of the chemistry of this heterogeneous group of substances.

Humic substances are affected by a great number of biological and chemical transformation mechanisms, including microbial processes and photochemical degradations reactions. The papers in Chapter 2 demonstrate the greatly diversified character of this subject.

In recent years, the effect of naturally occurring matter on the mobility and distribution of man-made pollutants has attracted particular attention. Several studies have shown that the transport of toxic, hydrophobic organic substances in soil/water systems can be facilitated by the presence of humic substances. An even larger number of studies have dealt with complexation of metals and sorption of metals to geological materials in the presence or absence of humic substances. Despite the accumulating amount of empirical data concerning the complexing properties of humics, the detailed description of these phenomena is still a matter of controversy. As discussed by Ephraim (Chapter 3), the heterogeneity and the polyelectrolyte character of the humic substances was not properly considered until a few years ago, and many problems remain unsolved.

The review by Petersen (Chapter 4) demonstrates that humic substances may have both beneficial and detrimental effects on the environment. Furthermore, biological effects of humic substances are often a result of interactions with other substances. The complex relationship between the toxicity of aluminium and the presence of humic substances can illustrate this fact.

It has already been mentioned that humic substances can act as precursors of toxic substances during drinking water disinfection. Further details of this type of interaction are given in the review by Holmbom (Chapter 5). Halogenation by-products can also be formed naturally, and humic substances may play an important role in the formation of substances normally considered to be xenobiotic.

Considering that numerous pollution problems and biological processes that can be affected or modified due to the presence of humic substances, it is of great interest to note that there are clear indications of upward trends in the concentration of humic substances in e.g. Swedish rivers. This provides another example of the crucial role of humic substances in the understanding of the present state and current trends in water and soil quality.

The articles published in this book demonstrate how humic substances now attract the attention of scientists from a large number of different disciplines. Several of the studied problems are of such a character that they can only be solved through multidisciplinary work. At the same time, numerous basic studies of humic substances and other natural organics are still being performed and are prerequisites for a better understanding of the role of these compounds in the environment.

Session 1:
Isolation, Fractionation, and Characterization

Factors to be Considered in the Isolation and Characterization of Aquatic Humic Substances

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Abstract

A detailed procedure using XAD-8 resin is presented for the isolation of dissolved fulvic acids and humic acids from water. The procedure entails pressure filtration to remove suspended sediment, sorption of humic substances onto XAD-8 resin at pH 2, desorption of humic substances in base, fulvic/humic separation at pH 1, desalting on XAD-8 resin, hydrogen saturation on cation exchange resin, and freeze-drying. Careful attention must be given to thorough resin cleaning and many procedural details in order to obtain relatively ash-free humic isolates. The equipment required for the procedure is expensive and the method is time consuming, but no other isolation method is known to produce quantitative and unaltered humic isolates from water. The procedure can be used to isolate small quantities (less than 100 mg) of humic substances from water, or it can be scaled to produce large quantities (100 g or more) of humic substances from water. Humic substances may be characterized by several methods. The more useful traditional characterization methods include elemental analysis, ash content, functional group analysis by titration and infrared spectroscopy, and molecular weight analysis. The new characterization methods of ^{1}H-NMR, ^{13}C-NMR, pyrolysis/mass spectroscopy, amino acid analysis, saccharide analysis, and carbon isotopic analysis (^{14}C and δ ^{13}C content) are usually more definitive than traditional characterizations.

Introduction

The isolation of humic substances from water has progressed rapidly during the last two decades. Previous to 1970, few studies were conducted on aquatic humic substances because isolation and concentration procedures were poorly developed. Humic substances could be sorbed from water onto charcoal and anion exchange resins [1], but recovery of humic substances from these sorbents was low. To obtain isolates containing high concentrations of aquatic humic substances, some investigators used freeze-drying of selected, colored, whole river waters, such as the Sopchoppy and Suwannee River waters [2], which contained high concentrations of humic substances and low concentrations of inorganic suspended sediments.

The use of XAD resins to concentrate humic substances from water has been a major breakthrough in organic hydrology. These resins were first used by a research group at Iowa State University in the early 1970's [3-5] for isolation of trace organic contaminants from groundwater. Riley and Taylor [6] and Mantoura and Riley [7] were the first to use XAD-2 resin to isolate humic substances from water. During the middle 1970's the organic geochemistry researchers of the U.S. Geological Survey in Denver, Colorado, evaluated several XAD resins, studied in depth their sorptive behavior, and developed several procedures for isolating organic constituents, including humic substances, from water [8-13]. Other resins such as Duolite A-7 [14] and DEAE cellulose [15] have recently been advocated for isolation of humic substances from water.

XAD-2 and XAD-8 resins have been the two most popular resins used to isolate humic substances from water. After extensive comparative studies, Aiken, *et al.* [12] concluded that XAD-8 was a better resin than XAD-2 for the isolation of humic substances from water because the smaller pores of the XAD-2 resin excluded or were fouled by the relatively large humic solutes and that complete recovery of the sorbed humic substances could not be achieved. For these and other reasons, XAD-8 has been the resin of choice by most freshwater organic hydrologists. XAD-2 resin has continued to be the resin of choice by most marine hydrologists and some freshwater organic chemists and hydrologists.

Although the use of both XAD-8 and XAD-2 resins has proliferated during the last decade, it is the author's opinion that inadequate attention has been given by investigators to limitations in the usage of the resins. The purposes of this paper are 1) to reiterate the general criteria and specific chromatographic principles which must be considered by the user of XAD and other resins, 2) to present a detailed procedure for isolating humic substances from water, 3) to provide detailed information essential to those researchers planning a humic substances isolation effort, 4) to make the reader aware of the cost and scale of humic substances isolation procedures, and 5) to emphasize the necessity for following detailed and time-consuming procedures which are required when using XAD resins if one is to achieve exacting research findings and characterizations of aquatic humic substances.

Materials and Methods

Resins

XAD-2 and XAD-4 resins are styrene-divinylbenzene copolymers. The resins are macroporous of low polarity with a 20 to 50 mesh size. XAD-2 resin has less crosslinkage, larger pore size, and less surface area than XAD-4 resin. Both resins can be obtained from Rohm and Haas Company, 500 Richmond Street, Philadelphia, PA 19137.[1]

XAD-8 resin is an acrylic ester polymer of intermediate polarity. It is macroporous with a 20 to 50 mesh size. It has a larger hydrated pore size and less surface area than XAD-2 resin. The resin can also be obtained from Rohm and Haas.

Duolite A-7 is a weak base, macroporous, anionic-exchange resin which is a phenol-formaldehyde condensation product. The resin can be obtained from Diamond Shamrock Chemical Company, Nopco Chemical Division, 1901 Spring Street, Redwood City, CA 94063.[1]

AG SOW-X8, a non-macroporous cation exchange resin, was obtained from Bio-Rad Laboratories.[1] The resin is 20 to 50 mesh size and is in the hydrogen form.

Stainless-Steel Canisters

If the filtration and water processing is not done in a streamside mobile trailer, 40-l stainless-steel cans are convenient to transport water to the laboratory site. Several dozen of these cans are desirable for this use.

Glassware

Soxhlet Extraction Apparatus of sufficient size to hold 2 l or larger extraction thimbles. Such very large extractors will have a solvent reservoir capacity of 12 to 15 l and stand 1.5 to 2 m in height. Three of four units are desirable.

Glenco glass columns, 3500 series with Teflon fittings and tubing. Column sizes for XAD resin adsorption chromatography are 12x125 cm and 8x90 cm. Column sizes for cation exchange chromatography are 3x60 cm for the cation-exchange resin precolumn and 6x100 cm for the final cation-exchange columns.

Glass jugs of 19 l and 45 l capacity made of Pyrex or Kimex hard glass. Twenty-five units of each size are desirable.

Filtration Apparatus

Millipore stainless-steel plate filter apparatus that holds 142 mm or 293 mm membrane filters. Twenty-four or more units of 142 mm size, or six units or more of 293 mm size, or equivalent. One of the 293 mm units has an equivalent filtration surface area of four of the 142 mm units.

Membrane filter of 142 mm or 293 mm in diameter, 0.45 μm porosity, and free of organic detergent wetting agents. Gelman vinyl-metricel membrane filters #64835 (142 mm in size) and #64838 (293 mm in size) or equivalent are required.

Stainless-steel filtration reservoir tanks of 20 l to 60 l capacity that are capable of being pressurized to 200 psi are required to provide a water supply for every two to four stainless-steel plate firers. A very large stainless-steel tank of capacity (in excess of 100 1) may be desirable. The reservoir tank is connected to the individual

[1]Use of any trade names is for descriptive purposes only and does not constitute endorsement by the U.S. Geological Survey

plate filters by flexible stainless-steel tubing with quick-disconnect fittings on each end.

Nitrogen or helium gas for pressure filtration should be of high quality. Gas pressure for filtration may be accomplished by several small gas bottles or a few large gas bottles with a manifold to each pressure reservoir.

Instrumentation

- A peristaltic pump (Cole-Parmer Masterflex) is needed to deliver filtered water and elution reagents to the resin columns.

- A Homelite AP 220 positive displacement pump with stainless-steel heads can be used to deliver the water from streams to 40 l stainless-steel cans for water transport.

- A portable pH meter and a conductivity meter with flow-through cells are desirable.

- A DOC Carbon Analyzer such as a Beckman 915 or equivalent.

- A large capacity freeze-drier that can freeze-dry 8 to 12 l of liquid each day.

Two types of centrifuges are desirable. One should be a large-capacity, low-speed unit such as an International Model K or equivalent. A second unit should be of moderate capacity and high speed such as can be achieved with a swing-out bucket head on a Sorval RC-2B centrifuge.

Reagents

Reagent grade pellets of NaOH or higher purity. Reagent grade HCl or higher purity. Reagent water with a low organic carbon concentration and specific conductance less than 0.5 S. If reagent water contains a DOC concentration of 0.5 mg C/l or higher, the water should be purified by passage through a column of XAD-8 resin before use.

Occurrence and Abundance of Dissolved Aquatic Humic Substances

Dissolved humic substances are only part of the dissolved organic carbon (DOC) in water and are never equivalent to the total DOC. Because natural waters contain adiversity of dissolved and particulate inorganic and organic constituents, and many of the dissolved organic constituents are not humic substances, water must be filtered and the dissolved humic substances selectively removed. Dissolved humic substances include only dissolved fulvic and humic acids; there is no dissolved humin fraction. Dissolved fulvic and humic acids in uncolored freshwater streams commonly

comprise approximately 40 percent and 4.5 percent, respectively, of the DOC content of 3 to 6 mg C/l [16,17]. The ratio of fulvic acids to humic acids is commonly 9:1. In organically colored waters common to Canada, Scandinavia, and Northern Russia, humic substances as a percentage of the DOC increase with increasing DOC concentrations, and often account for 60 to 80 percent of the DOC. In these highly colored waters, the humic acids become a much higher percentage of the DOC and the fulvic acids to humic acids ratio commonly decreases to 4:1 or lower.

Analyses of approximately 100 surface water samples of the United States yield an average of general constituents according to a sixfold DOC fractionation procedure [17] as shown in Fig. 1. Approximately 99+ percent of the DOC are natural constituents and less than 1 percent are contaminants.

The average DOC concentration in potable groundwaters is 1 mg C/l [18]. The average DOC distribution of approximately 25 groundwater samples collected from aquifers in the United States for the same six organic fractions is shown in Fig. 2. Essentially 100 percent of the groundwater DOC are natural organic constituents, although in infrequent instances of groundwater contamination, the percentage reported as contaminants is commonly high and extremely variable. The DOC concentration in groundwaters is much lower than in surface waters, and the organic constituents as part of the total DOC are also very different. For example, humic substances are commonly less than 20 percent of the groundwater DOC; whereas, low-molecular-weight organic acids may comprise 50 percent of the DOC.

The DOC of deep uncontaminated sewater has generally been reported to be near 1 mg C/l [19,20]. The concentration of humic substances in seawater, which is primarily as fulvic acid, is 10 to 20 percent of the DOC. The DOC of deep seawaters is lower (1 mg C/l or lower) than seawater in the euphotic zone or near coastal regions (1.5 mg C/l or higher). The precise DOC value for seawaters is now in question because it has been found to be method dependent [21] with an underestimation of the low-molecular-weight hydrophilic acid fraction.

Choice of Resin for the Concentration of Humic Substances From Water

Several isolation methods, including freeze-concentration of whole water samples [22], freeze-drying of whole water samples [2], strong anion-exchange resins [1], charcoal [23], and flocculation with heavy metal oxides and hydroxides [24-27], have been used in attempts to isolate humic substances from water. These methods were of limited success because only partial isolation and/or recovery of humic substances was achieved, the humic isolates were mixed with many non-humic organic solutes and inorganic constituents (clay minerals and inorganic salts), and because various contaminants were introduced into humic isolates. After the initial usage of XAD resins during the 1970's, it was recognized that XAD resins held great promise in overcoming the limitations of previously used methods for isolation of humic

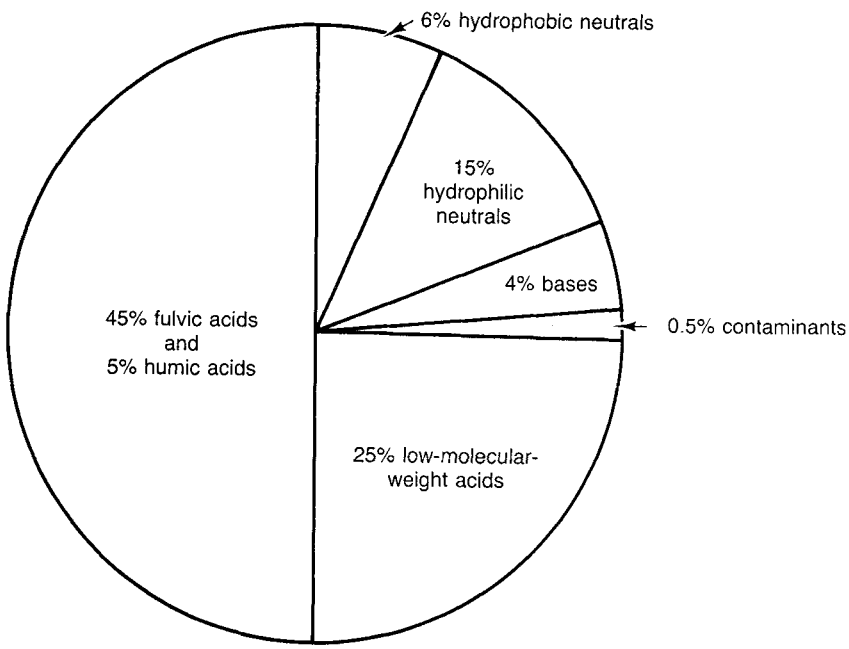

Figure 1 Distribution of surface water DOC in rivers of the United States.

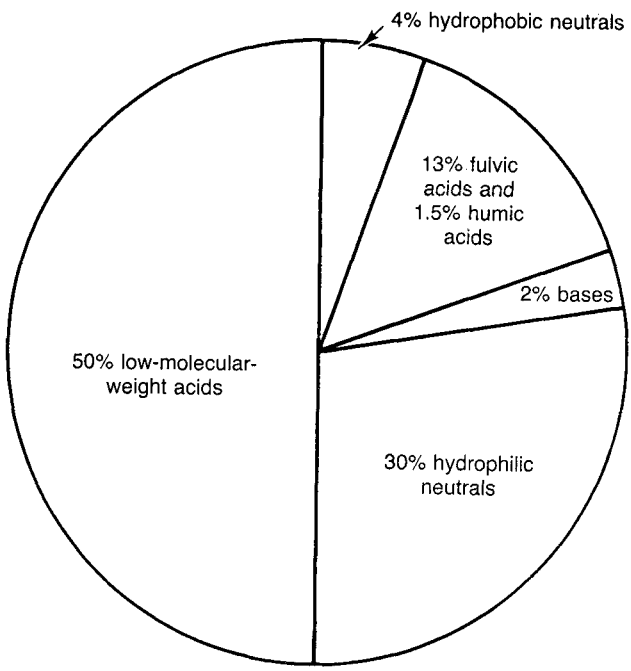

Figure 2 Distribution of groundwater DOC in aquifers of the United States.

substances from water. However, before XAD resins could be advocated, many basic and applied experiments on their sorptive properties had to be conducted.

All the XAD resins are macroporous and uncharged, but they vary in composition, surface area, pore size, cross-linkage, density, and polarity as shown in Table 1. XAD-1, XAD-2, and XAD-4 are aromatic polymer matrices of styrene-divinylbenzene, whereas XAD-7 and XAD-8 are aliphatic polymer matrices of methylacrylic acid (acrylic ester). Because of the high surface area of XAD-4 resin, it was postulated to be the best resin for isolating humic substances from water. Its high surface area was expected to result in a high sorptive capacity.

Table 1 Composition, surface area, pore size, cross-linkage, density and polarity of XAD resin [28].

XAD resin	Surface Area m^2/g	Pore Size Å	Cross-linkage	Density	Polarity
		Styrene-divinylbenzene polymer			
1	100	200	low	1.06	none
2	330	90	moderate	1.08	none
4	750	50	high	1.09	none
		Acrylic ester polymer			
7	450	80	moderate	1.25	slight
8	250	250	low	1.26	slight

Several systematic experiments [8-14] were conducted to elucidate the properties of the resins, their sorptive capacities, the rates of solute sorption, the mechanisms of solute sorption, the effects of solute size on sorption, and many other factors of resin usage. Several of the experimental results were not predicted.

Some of the more important results and conclusions are listed as follows:

1. Excessive resin bleed precluded the use of XAD-7 for concentration of humic substances from water.

2. The testing of XAD-1 was discontinued because its manufacture was discontinued by Rohm and Haas.

3. In experiments with solutions of small organic solutes, the ranking of resin sorptive capacities was XAD-4 > XAD-2 > XAD-8.

4. In experiments with natural waters and solutions of humic substances, the ranking of resin sorptive capacities was XAD-8 > XAD-2 > XAD-4.

5. Due to the large size of humic solutes dissolved in natural waters, the small pores of XAD-4 and XAD-2 excluded humic substances from a large part of the interior macroporous surface of the resin beads. Many macropores were plugged with humic substances, preventing sorption of smaller organic solutes.

6. The recovery of humic solutes from XAD-2 and XAD-4 resins was incomplete, ranging from 75 percent to 85 percent. This poor recovery was postulated to be due to the formation of π-π bonds between the aromatic matrix of these resins and aromatic moieties within the humic substances which lead to an inadvertant fractionation of the humic substances from water.

7. The sorptive rate for humic solutes was most rapid on XAD-8; it exhibited the highest sorptive capacity for humic solutes, and the XAD-8 resin yielded essentially 100 percent recovery of sorbed humic substances with no specific sorption.

The pore size of the hydrated XAD-8 resin is in excess of 250 Å, thus enabling the complete access of its surface area to sorption of natural organic solutes. The theoretically higher surface areas are not used nor is the higher potential sorptive capacities of XAD-2 and XAD-4 achieved in isolation of organic solutes from natural waters because the small pores of the resin are plugged by high-molecular-weight naturally occurring humic solutes that limit the access of other natural organic solutes to a large part of the inner macroporous surface sorptive area [12]. For these and other reasons, XAD-8 is the resin of choice for the isolation of humic substances from natural waters.

The author has performed similar experiments to evaluate XAD-2 and XAD-8 resins for the isolation of dissolved seawater humic substances (primarily fulvic acids). The unpublished results were not as definitive as with freshwater humic substances. The XAD-2 and XAD-8 resins appeared to be almost equally effective in isolation of dissolved seawater humic substances, but there were some compositional differences between humic substances isolated on XAD-2 resin and XAD-8 resin.

Overview of Resin Isolation Procedure

The resin procedure to obtain fulvic acid and humic acid isolates from water can be referred to in two parts. The first part (isolation and concentration of humic substances from water) entails filtration, acidification, resin sorption, resin elution, and resin column regeneration. After a water-sampling site is chosen according to research objectives, a representative sample of the water source is collected into suitable containers for transport to the laboratory, or the water can be pumped continuously (at the sampling site) into a mobile laboratory designed to contain the equipment necessary for water processing. The water sample is pressure filtered through a 0.45 µm membrane filter to remove particulate material. The filtered water is collected in 45 l glass jugs and acidified to pH 2 in order to protonate the humic substances to an uncharged state. The acidified water is pumped onto a preparative

glass column containing several liters of cleaned XAD-8 resin. The uncharged humic substances and the neutral organic solutes are sorbed onto the resin column until the column is near saturation and breakthrough of the humic substances occurs in the column effluent. The capacity of the column to concentrate humic substances or other given solutes from water is referred to as k', the column distribution coefficient. A k' of 50 will result in approximately 95+ percent of the humic substances in water being retained on the XAD-8 resin column. For most uncolored stream waters, 306 l of filtered water can be processed through the column before saturation of a 9 l resin column occurs. After saturation, the resin column is flushed with 0.1 M NaOH, the humic substances are ionized, and then eluted from the column in three bed volumes of basic solution. The eluted humic substances are acidified to a pH between 4 and 5, and stored for additional processing. The resin column is acidified to pH 2 with HCl. The process of adsorbing and eluting the humic substances from a given quantity of water (usually 306 l) at a desired k', and the reacidification of the resin column, which is made ready to accept another amount of acidified filtered water, is referred to as "a run" or "one run" in sample isolation and concentration.

The second part of the procedure (humic substances separation and purification) entails the separation of fulvic acids from humic acids, desalting, and hydrogen saturation of humic substances. Humic substances that have been eluted from a number of runs are combined and the pH lowered to 1 with HCl. The humic acids will precipitate; the fulvic acids will remain in solution. Humic acids are separated from fulvic acids by high-speed centrifugation. The fulvic acids are readsorbed onto the XAD-8 resin column and washed with deionized water to remove the inorganic acid. The fulvic acids are back eluted from the resin column with 0.1 M NaOH, the eluate is hydrogen saturated by passage through a column of hydrogen-saturated exchange resin, then freeze-dried. The humic acid is dissolved in dilute NaOH, diluted to a concentration less than 250 mg C/l, acidified to pH 2.0 with HCl, desalted on the XAD-8 resin column the same as the fulvic acids, hydrogen saturated by resin exchange, and then freeze-dried.

Procedural Details and Considerations

The resin isolation procedure for aquatic humic substances is very lengthy and time consuming. There are many details and helpful hints, which if followed, make the procedure more successful, more trouble free, and easier to accomplish. Some of these details will be incorporated in this section along with some of the reasons for various operations involved in the procedure.

Cleaning the Resins

The proper and complete cleaning of the resins for organic solute isolation is as important as resin selection and usage for the success of the proposed research objective. Inadequately and improperly cleaned resin can negate all the time and effort in organic isolation and fractionation due to extensive artifacts and resin contamination in the humic isolates.

An extensive amount of time, effort, and expense is required for adequate resin cleaning. There are no shortcuts; several weeks or months are required for cleaning resins before they can be used for solute isolation.

For efficient time and productive effort, 5 to 20 l of clean resin is required for humic substances isolation from each site. The cleaning of 20 l of resin is not a small task. It requires days of planning, the purchase of special equipment, and a period of months for actual resin cleaning in several solvents.

The resins, as received from the supplier, are commercial grade with an abundance of contaminants and unpolymerized monomers. The resins are also of nominal mesh size and have an abundance of smaller sizes (fines). The first step in the cleaning process is to wash the resin in a large container with 0.1 M NaOH solution. The NaOH solution should be renewed daily for the first 10 days. After the NaOH solution is decanted each day, the resin should be washed several times with deionized water with decantation of the fine resin particles. The removal of fine particles is essential for maintenance of a high flow rate through large resin columns. Otherwise the columns will become clogged with fine resin particles and adequate flow rates can not be maintained. After the 10-day cleaning in NaOH, and the subsequent rinsing with deionized water, rinse the resin three times with methanol. The resin is now ready to be transferred to a Soxhlet cleaning apparatus.

Using three or four Soxhlet units, the solvent cleaning of the resin can be done in an assembly-line process. The extraction thimbles containing resin can be moved in sequence from one solvent to another after each 5-day cleaning period. The recommended cleaning sequence is methanol, acetonitrile, and diethyl ether. Five days in each of these solvents is considered to be one cleaning cycle. The resins can be cleaned in additional solvents, if desired, and should be cleaned in any solvent that will be used to elute sorbed solutes from the resin in an isolation procedure.

For new or unused resin, it is imperative that the resin be cleaned a minimum of two and preferably three cleaning cycles before use because the new resin has a large quantity of impurities. Finally, after cleaning the resin, it should be cleaned once more for two days in a Soxhlet containing methanol and then stored in methanol before use.

Used resin which has been used in organic solute isolation should be saved and cleaned for a minimum of one and preferably two cleaning cycle(s) if time permits before the next usage. It is usually much more desirable to clean and reuse the resin than to start with new or unused resin because the resin becomes cleaner with repeated cleaning cycles and the cleaning with NaOH is not necessary.

Prior to resin usage, the resin should be slurry packed into a glass chromatographic column fitted with Teflon endcap, valves, and tubing. The column should be filled to approximately 90 percent capacity. The resin column must be rinsed with approximately 75 to 100 column volumes of distilled (reagent) water to free the resin of methanol. The final effluent washings of the resin column should be

tested for DOC and should contain no higher DOC concentration than is normal for distilled water (<0.5 mg C/l). The column must be rinsed in excess of nasal detection of methanol. It is imperative that the resin is washed free of methanol because the sorptive properties of the resin are decreased for most solutes in water-methanol mixtures.

After the methanol has been rinsed from the resin, the resin should be base rinsed with one column volume of 0.1 M NaOH and then acid rinsed with one column volume of 0.1 M HCl in order to clean the resin of any hydrophobic solute contaminants in the large volume of distilled water. This base-acid rinse should be repeated immediately before column use. This base-acid rinse should be repeated before resin usage during any period which the resin has been standing unused in acid solution for 1 day or longer in order to clean the resin of any solubilized or hydrolyzed resin components.

The XAD resins should not be stored or left standing in basic solution for extended periods because the resin will slowly hydrolyze and contribute resin bleed to organic isolates desorbed from the resin. Between daily usage of the resin, it should be stored in acid solution. The time required for elution of the resins in basic solution should be minimal to prevent resin bleed into the desorbed organic solutes.

Volume of Water Sample

For practical purposes, a minimum of 100 to 200 mg of humic substances is required for adequate characterization. The amount needed in addition to characterization requirements for given proposed research objectives vary greatly, but 150 to 200 mg are usual minimal quantities. Therefore, a total minimal quantity of 300 to 400 mg is required. For surface water with a DOC of 4.5 mg/l, assuming 40 percent of the DOC as fulvic acids, and assuming an anticipated recovery of 80 percent, approximately 275 l of water must be processed to acquire 400 mg of fulvic acids. To acquire 400 mg of humic acids from the same water sample would require approximately 9 times the amount of water to be processed (approximately 2500 l).

Isolation of 300 mg of humic acids from groundwaters requires the processing of a very large volume of water. For example, groundwater with a typical DOC of 0.5 mg/l, containing 3 percent of the DOC as humic acids, and anticipating an 80 percent recovery of humic acids, would require the processing of 25,000 l of water.

The isolation of 300 mg of seawater humic acids seems completely impractical based on the experience of the author. After processing over 32,000 l of seawater, less than 50 mg of ash-free humic acids were isolated. In order to isolate 300 mg of seawater humic acids, approximately 200,000 l of seawater must be sampled and processed. It is a much more reasonable effort to isolate 300 mg of fulvic acids from seawater. Assuming a DOC of 1 mg/l, 10 percent of the DOC as fulvic acids, and 80 percent recovery, approximately 4,000 l of seawater must be processed.

Sampling-Site Selection and Water Sampling

The water-sampling site is often dictated by specific research objectives. If there is some liberty in site selection, it should be free from local influences of contamination and far enough downstream from the confluence of another stream that complete mixing within the stream has occurred. Stream sampling should be the composite of several vertical samples across the stream transect rather than point sampling or sampling from the streambarlk.

When sampling wells, one should empty the casing several times to remove stagnant water before sample collection. To prevent sample contamination from oil-lubricated pumps, one should sample only wells with artesian flow or wells containing water-lubricated pumps.

The sampling of seawater is not an easy task because special equipment is often needed for sampling from ocean-going vessels. Care should be taken to adequately document the sampling depth and location because samples taken from nearshore and shallow depths in the upper euthrophic zone may be greatly influenced by algal and bacterial growth. Because seawater is corrosive to stainless-steel vessels, saline samples should be collected and stored in glass, glass-lined, or Teflon-lined containers.

Filtration of Water Samples

Separation of the dissolved and particulate phases in water is essential for removal of particulate inorganic sediments, particulate organic detritus, and various microorganisms and aquatic invertebrates. It is also well recognized that particulate and dissolved organic substances differ in composition and reactivity. The most universally accepted definition for separation of dissolved and particulate constituents in water is filtration through a 0.45-μm membrane filter. This definition is arbitrary as are other techniques such as centrifugation and density separation. The author's recommendation for separation of dissolved humic substances from whole water samples is filtration through a "partially clogged" non-contaminating 0.45 μm membrane filter. One such membrane filter is the Gelman Metricel without a detergent wetting agent. In the past, this type of membrane filter was only supplied containing an organic detergent as a wetting agent for the filter. The detergent contaminated the filtered water sample. Preleaching the filter to remove the detergent was difficult and time consuming. This type of membrane filter will selectively sorb organic solutes of very low water solubility, but it has a very low capacity for humic or fulvic acids. The ideal membrane filter is not yet available; therefore, knowledgeable choices must be made for water filtration according to the specific objectives of the proposed research. Silver membrane filters were frequently used for filtration in the past before the non-detergent Gelman Metricel membrane filters were available. Silver membrane filters are desirable because they have carefully controlled pore sizes and do not sorb organic substances from water. These membranes are expensive and have been found to result in slight contamination of humic substances

with Ag, Hg, and Fe from the membrane filter. Such trace metal contamination may limit humic isolates for trace metal studies.

The major purpose of filtration is to obtain the dissolved organic solutes free of inorganic and organic particulate constituents in the water. Most natural water samples appear to contain a continuum in size of both organic and inorganic constituents from true solution to large particulates. It is often difficult to determine if humic substances are in true solution, micelles, or small aggregates. Clay and silt size particles of crystalline and amorphous minerals are always suspended in water. Their presence in humic isolates results in high ash contents of humic substances and causes numerous interferences with organic analysis; therefore, the best index of filtration effectiveness is low inorganic ash contents. Because many clay-size minerals such as smectites and amorphous oxides are in the size range of 0.2 μm to 0.02 μm, and will readily pass through a 0.45 μm filter, a "clogged" 0.45 μm membrane filter is advocated for water filtration. The "clogged" 0.45 μm membrane filter will effectively remove essentially all the fine colloidal clay particles; therefore, the effective pore size of the "clogged" 0.45 μm membrane filter is in the size range of 0.01 μm or less. In practical use, the "clogged" condition is attained by passing sufficient amounts of water sample through the 0.45 μm filter at a pressure of 5 to 10 psi to reduce the flow rate to a dropwise rate. The period of time usually required to clog the filter is 10 to 15 minutes. The water that passed through the membrane filter during the clogging process is either discarded or refiltered through the clogged membrane filter.

Humic isolates that are obtained after membrane filtration and desalting by cation exchange are routinely low in ash content. Fulvic acids are commonly less than 1 percent ash and humic acids are commonly less than 2.5 percent ash. Filtration almost always obviates the necessity for treatment of humic substances with mixed HCl/HF solutions to dissolve inorganic ash constituents. The HCl/HF treatment is undesirable and should be avoided because of fractionation and large losses of humic substances during the treatment.

Resin Sorption and Desorption of Humic Substances

Filtered water in 45 l glass jugs is acidified to pH 2.0 with concentrated HCl. The acidified water sample is delivered to the top of the 9 l resin column by a peristaltic pump (Cole-Palmer Masterflex) with a number 18 roller head at a rate of 1 l/minute. All the connecting lines and valves are Teflon except for the 20-cm portion of tubing in the roller head; that portion is Tygon. According to Equation (1) [29],

$$V_E = V_O (1 + k') \qquad (1)$$

a k' of 50 can be achieved with the passage of 306 l of water. V_E is the breakthrough elution volume of a solute such as fulvic acid, V_O is the void volume of the resin column, and k' is the column distribution coefficient or column capacity factor.

$K' = \frac{\text{(mass of solute sorbed on XAD-8 resin column)}}{\text{(mass of solute dissolved in column pore water)}}$ (2)

After 306 l of acidified water is passed through the column, the pump is stopped and the inlet and outlet tubings on the glass column are reversed for back elution of the resin column. Care must be taken to fill the inlet line by pumping with 0.1 M NaOH before connecting the tubing to the bottom connection of the column. Open the valve at the bottom of the column simultaneously at the beginning of pumping the 0.1 M NaOH. The initial pumping rate must be near the maximum pumping capacity of the pump head so that the resin column will be lifted up to the top of the glass column, thus removing the void space at the top of the glass column. After the resin column has moved upward to the top of the glass column, reduce the pumping rate to 400 to 500 ml/min or to that rate which will support the resin column in the top of the glass column. The 0.1 M NaOH elution solution should move slowly and uniformly up the resin column. If the air in the base inlet line were not initially removed, the introduction of air bubbles into the bottom of the column usually would cause channeling of the base flow and occasional overturning of the resin column during elution.

The elution technique enables the collection of a highly concentrated center part or "center cut" of the humic substances elution in a 1 l volume. The humic substances in this part will be the most concentrated and may not require further concentration if the concentration is greater than 500 mg C/l. This part of the eluate should be acidified immediately to a pH between 3 to 4 and stored on ice.

An idealized back-elution curve for humic substances eluted with 0.1 M NaOH from a 9 l column of XAD-8 resin is shown in Fig. 3. The sequential segments of the elution curve are designated as A through E. Color may be observed in the eluate just before the 6 l void volume of the column is attained, even though the pH of the eluate is below pH 7, due to slight channeling of base in the resin column. This 6-1 portion of the elution curve is Part A. The color, concentration of eluted humic substances, and the pH of the eluate will gradually increase until the eluate becomes strongly basic (> pH 12) in the second portion of the elution curve from 6 l to 7.5 l. This 1.5 l portion is Part B of the elution curve. Part C of the elution curve is the 1 l "center-cut" previously mentioned. The color and concentration of humic substances gradually decreases in the fourth portion (Part D) of the elution curve from 8.5 l to 14 l. At the 14 l point in the elution curve, the pumping of base is stopped and the pumping of 0.1 M HCl is begun by rapidly changing the inlet pump tube from base to acid containers. In the next portion of the elution curve (Part E) the concentration of humic substances becomes very low and approaches zero after the resin column becomes strongly acidic.

The eluate from Parts A through E of the elution curve are treated in three different ways: stored for separation of fulvic and humic acids, stored for reconcentration, or added to the next column run. The 1.5 l Part B of the eluate prior to the "center-cut" (Part C) and the 5.5 l Part D after the "center-cut" should be combined in a separate glass container, the pH lowered with HCl to a pH between 3

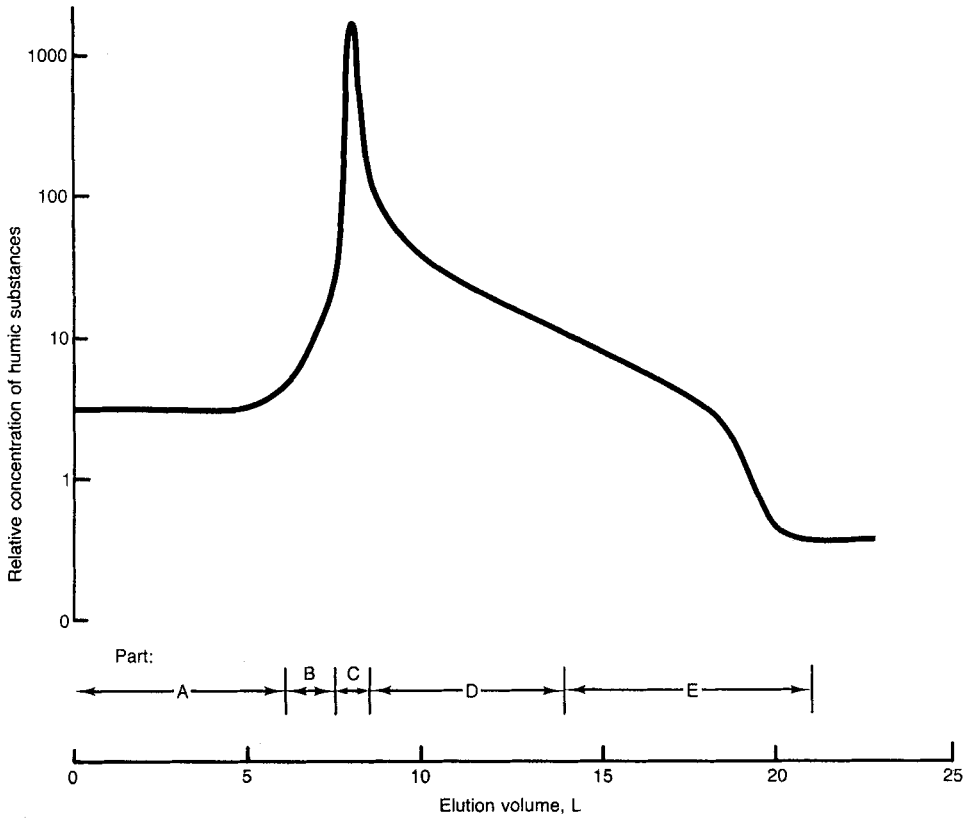

Figure 3 Idealized back-elution curve of humic substances with 0.1 M NaOH from a 9 l column of XAD-8 resin.

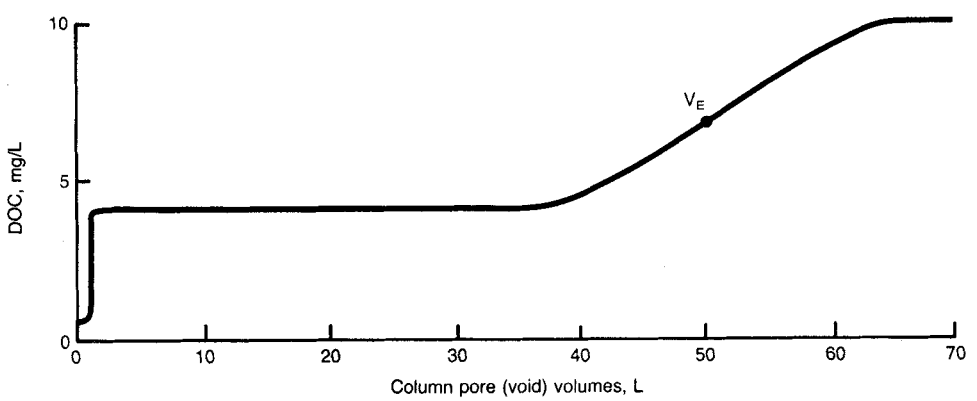

Figure 4 Idealized breakthrough curve for stream humic substances on XAD-8 resin (sample DOC of 10 mg/l, hydrophilic DOC of 4 mg/l and humic substances are the primary constituents of hydrophobic DOC. V_E is the sample elution volume).

and 4, and stored in an ice bath for later reconcentration. The "center-cut" (Part C), the 1.5 l Part B, and the 5.5 l Part D are designated as retained parts of the elution. The void volume during elution (Part A) prior to the retained parts and (Part E) until the eluate becomes strongly acidic (approximately between pH of 1 and 2) are combined, acidified to pH 2, and added to the 306 l filtered sample for the next column run.

After several column runs, the accumulated "other-than-center-cut" concentrated organic solutes (Parts B and D) are reconcentrated as a separate run on the large XAD-8 resin column. The final reconcentrations are conducted on smaller columns of XAD-8 resin. Virtually all of the humic substances are adsorbed upon reconcentration with little or no losses. During the initial concentration and elution of humic substances in some samples, the concentration of humic substances may be so low that the "center-cut" (Part C) of the elution is less than 500 mg C/l. In these instances, Part C should be combined with Parts B and D and treated accordingly.

Separation of Fulvic and Humic Acids

All the elution "center cuts" and the reconcentrated humic substances are mixed in a 45 l glass container and acidified to pH 1.0 with concentrated HCl. In the well-homogenized concentrate, a carbon concentration of 500 mg/l is minimal for rapid and complete precipitation of humic acids. The concentrated sample should be chilled to 2°C in an ice bath, the flocculated humic acids resuspended several times over a 12-hour period, and then allowed to settle. The precipitated humic acids are separated from the soluble fulvic acids by centrifugation.

Desalting, Hydrogen Saturation, and Freeze-Drying

The solution of fulvic acids contains high concentrations of sodium and HCl. The major part of these ions and salts is removed by desalting on an XAD-8 resin column. Fulvic acids at pH 1.0 should be pumped slowly (400 to 500 ml/min) onto a 9 l column of XAD-8 resin until the observed color of the sorbed fulvic acids extends approximately one-third down the length of the column; at this time, pumping of fulvic acid should cease and pumping of distilled water should begin. Specific-conductance monitoring of the effluent also is initiated using a flow-through cell. During leaching of the acidic salt solution from the void volume of the column, the specific conductance decreases rapidly. Concurrently with acid removal, the pH increases, and the fulvic acid begins to move slowly down the column. The column should be rinsed with distilled water until the specific conductance is decreased to 250 µS/cm. The XAD-8 resin column is then back-eluted with 4 column volumes of 0.1 M NaOH. Color, due to fulvic acids, is normally observed to elute from the column during rinsing when the specific conductance decreases to less than 700 to 800 µS/cm. These colored washings, collected until the specific conductance decreases to 250 µS/cm, should be acidified and added to the next desalting run.

The XAD-8 resin column should be back-eluted rapidly with 0.1 M NaOH, the dilute first part of the back eluate mixed with the initial concentrated basic part of

the elution, and the solution passed rapidly through a small precolumn (for example, 60 cm by 3.5 cm) of hydrogen-saturated exchange resin. This limits the contact time of fulvic acids with base to only a few minutes. The last part of the base eluate of fulvic acids is introduced directly into the cation-exchange resin column. The purpose of the precolumn is not to completely hydrogen saturate the fulvic acids, but to remove most of the sodium and quickly neutralize the basic solution, which should prevent oxidation of fulvic acids in basic solution. The solution of fulvic acids will be below pH 4.5 after the pretreatment. The solution of fulvic acids is then passed at a fast, dropwise rate through another hydrogen-saturated resin exchange column (for example, 100 cm by 10 cm) for complete hydrogen saturation of the fulvic acids. After complete hydrogen saturation, the sodium concentration should be less than 0.1 ppm. The purpose of mixing the first dilute part of the elution with the concentrated "center cut" part is to dilute the fulvic acids to less than 300 mg C/l. Sometimes fulvic solutions of carbon concentrations in excess of 300 mg C/l will precipitate on the acidic precolumn and will not hydrogen saturate normally. If precipitation occurs on the hydrogen-saturated precolumn of cation exchange resin, the column must be unpacked, rinsed with 0.1 M NaOH, the entire fulvic acid solution diluted with distilled water, and then precolumn treatment repeated.

A non-macroporous cation exchange resin is preferred over the macroporous type. The cation exchange capacity of both types is almost the same, but the macroporous type allows penetration of the fulvic acids into the beads and the end of the saturation is protracted with a long elution tail from the cation exchange column. This excess dilute solution requires more rotovap preconcentration before freeze-drying.

The precipitated humic acids are kept moist with distilled water until they are desalted and hydrogen saturated. To desalt the humic acids, they are solubilized in dilute NaOH and the DOC concentration adjusted to 250 mg/l as carbon or less. The solution is then adjusted to pH 2.0 and pumped slowly onto a large XAD-8 resin column. At this point, the procedure used for desalting the humic acids is the same as that for fulvic acids. To accomplish hydrogen saturation of the humic acids without precipitation in the cation-exchange resin column, it is imperative that the humic-acid concentration in solution not exceed 250 mg/l as carbon during passage through the cation exchange resin. Concentrations of humic acids in excess of 250 mg/l frequently precipitate and clog the cation exchange resin, necessitating a repeat of the desalting procedure.

The hydrogen-saturated fulvic acids and the hydrogen-saturated humic acids should be separately concentrated by vacuum rotovap at room temperature to approximately 1500 mg C/l to facilitate freeze-drying of reasonable volumes of humic solution. Care should be taken to maintain a high vacuum during freeze-drying to insure a light fluffy product. A poor vacuum will result in dark, hard crystal-like formation due to thawing and reconcentration. Air drying or oven drying should be avoided due to extreme denaturation and possible alteration of the humic samples.

Chromatographic Considerations

Resin columns do not have an infinite capacity for solute sorption. This concept has been the most difficult for many users of the resin technique to realize. It is counterproductive to ignore all capacity considerations and pump large amounts of water at a rapid rate through small columns of resin and expect solute recoveries to be representative. The capacity factor (k'), volumes of XAD-8 resin, and amounts of water from which 95+ percent recovery of a given solute can be concentrated, and void volumes of the resin columns are given in Eqns (1) and (2). After the determination of the volume of XAD-8 to be used, a column size can be selected to contain the resin plus approximately 5 to 10 percent additional volume. The unfilled 5 to 10 percent column volume above the resin bed is desirable for efficient column operation. It permits small volumes of air to enter the column without effecting the water flow in the resin column during solute sorption, the observation of slow leaks in plumbing of the column, the time lag necessary to stop the water flow in the column before air enters the column when the pump tubing periodically ruptures, and permits small volume changes in the packing of the resin during column elution and regeneration. The void volume of XAD-8 resin is approximately 65 percent per unit volume as determined experimentally by the breakthrough curve for unretained inorganic ions.

The k' distribution for natural organic solutes dissolved in water onto XAD-8 resin has been found to be a bimodal; low k' values of less than 5 to 10 are termed hydrophilic and higher k' values of greater than 40 are termed hydrophobic. Because of the sparcity of solutes in natural waters with k' values in the 10 to 40 region, there is a natural resolution between hydrophilic and hydrophobic solutes according to sorption on XAD-8 resin. The use of the terms hydrophilic and hydrophobic according to XAD-8 resin sorption can be somewhat ambiguous to the natural water system because all the dissolved organic solutes are hydrophilic in natural waters.

Humic substances have high k' values and are acidic in nature; therefore, they are designated as hydrophobic acids according to XAD-8 resin isolation. The k' values of all the colored, non-specific, carbonaceous organic substances in water which comprise the range of compounds called humic and fulvic acids in water are pH dependent with 99+ percent of these substances having a k' greater than 50 at pH 2 with less than 10 percent having a k' of 50 at pH 7. The components with a k' of 50 or greater at a pH of 7 are termed weak hydrophobic acids and are typically high in phenolic character. A strong hydrophilic acid fraction which remains ionized at pH 2 and will not sorb onto XAD-8 is usually very small (less than 1 to 2 percent of the total humic substances).

The k' of humic substances is slightly affected by concentration. The k' of humic substances in dilute solution and up to approximately 10 mg/l is essentially constant at 50 or greater. This k' is applicable for all uncolored waters and for colored water with a DOC up to approximately 15 mg/l. At higher DOC values and humic substance concentrations, the k' of humic substances (primarily fulvic acids) decreases. At DOC values of near 30 and 50 mg/l, the k' of fulvic acid decreases by

approximately 20 and 50 percent, respectively. The k' for humic substances on any size of column can easily be determined experimentally by plotting DOC versus the column pore (void) volumes of water sample passed through the resin column. After 1 column pore volume of sample input, there will be a constant breakthrough of hydrophilic sample DOC which will be approximately 40 percent of a surface water DOC value or approximately 70 to 80 percent of a groundwater DOC value. After approximately 40 column void volumes, the hydrophobic constituents of the DOC (primarily humic substances) will initially breakthrough with an increase in the breakthrough DOC. The humic substances breakthrough will increase as a typical S-shaped breakthrough curve until the DOC of the sample effluent is equal to the DOC of the sample influent. The breakthrough volume (V_E) at the midpoint of the S-shaped breakthrough curve is the value used to calculate the k' of humic substances in a given sample (Eqn 1). A curve of ultraviolet absorbance, fluorescence intensity, or any other quantitative parameters of humic substances versus column pore volumes can be used to determine the k' of humic substances. An idealized breakthrough curve for humic substances in water is shown in Fig. 4, in which the initial sample DOC is 10 mg/l, hydrophilic DOC of the sample is 4 mg/l, and the resin column pore (void) volume is 66 percent of the packed column volume.

The column capacity factor, k, is greatly affected by the flow rate of water through the resin column. Ideal sample flow rates have been determined to be between 5 and 10 column volumes per hour. For most organic solutes, k' decreases sharply with flow rates in excess of 10 to 15 column volumes per hour. At temperatures near room temperature (20°C), there are very small changes in k' with temperature fluctuations.

Column performance is seriously affected by fine particles of resin less than 60-mesh size. With time and usage of resin columns, the fine resin particles have a tendency to accumulate into a thin layer within the column. As this layer becomes thicker, it becomes more difficult to maintain adequate column flow rates without development of backpressure and subsequent leaks from column fittings. Thorough initial washing and decanting of resin fines is critical to efficient column operation.

Precautions in XAD-8 Resin Usage and Helpful Hints in Procedure Usage

As with any analytical method, certain precautions must be taken to assure quality results when using any resin including XAD-8. The magnitude of most of the potential problems of resin usage can be minimized or prevented if the resin is thoroughly cleaned initially. Inadequately cleaned resin will lead to problems in resin usage and in the organic isolates obtained by the resin procedure.

Long-term resin storage in solvents such as methanol, acetonitrile, acetone, or even water will result in slight resin dissolution or bleed. Most dissolution products can be removed by rinsing with several column volumes of high-quality distilled water, followed by rinsing alternately in dilute HCl and dilute NaOH. All XAD-8 resin columns should be rinsed alternately with dilute HCl and dilute NaOH daily before usage. Storage of bulk resin or of packed resin columns in water or dilute

acid should not occur for more than a few days in order to prevent microbial or algal growth on the resin. Immediately after the usage of a column of resin is completed, the column should be unpacked and the resin stored in methanol until it is cleaned by recycling in Soxhlet extractors. The time between resin use and recleaning should be minimal to prevent any microbial growth on or in the resin beads. The cleaned resin should be stored in methanol to prevent microbial growth, to prevent breaking of the beads during drying and storage, and to prevent the slacking of the beads (production of fines) upon drying and rehydration.

One precaution in XAD-8 resin usage must be adhered to, i.e., is do not store the resin in basic solution. The time periods during elution when the resin is in basic solution must be minimized to prevent resin hydrolysis which results in resin bleed and sample contamination. With continuous daily column use, do not leave the resin standing in basic solution overnight. After eluting the last sample concentration run of the day, the resin must be rinsed with dilute acid until the resin column effluent is acidic before leaving the column to stand overnight. XAD-8 resin is quite stable for short periods in basic solution, but hydrolysis of the resin increases with high pH (greater than 10) and with the length of time in basic solution.

To minimize any possibility of resin bleed in acidic solution, XAD-8 resin columns with sorbed humic acids should not be left standing overnight. The sorbed humic substances should be eluted and the column reacidified for overnight storage. Finally, recovery or elution of the sorbed organic solutes from the resin should not be attempted with any solvent that has not been used initially to clean the resin. All of the previous precautions apply for any special usage of XAD-2 or XAD-4 resins.

After the elution of adsorbed humic substances from the XAD resin column with 0.1 M NaOH at the end of each "column run", the NaOH is followed by rapid pumping of 0.1 M HCl until the column effluent is acidic to pH indicator paper. After stopping the pump, the column pumping is reversed for frontal or downward pumping through the resin column. With pumping ceased, the resin column will slowly repack itself from the bottom of the column upward. The repacking processes can be accelerated by gently shaking or jostling the glass column. During the repacking any small air bubbles or channels in the resin column are removed by upward flotation. After the resin column is repacked, one column void volume of 0.01 M HCl (a solution of pH 2) is pumped through the column before starting the next sample run.

In addition to saving time by reacidification of the resin column with 0.1 M HCl (pH 1) rather than 0.01 M HCl (pH 2), there is a tenfold savings of distilled water used to make acidic solutions. This savings of distilled water is especially useful in field operations. The next sample should not be used for acidification of the resin column because of possible losses of humic substances. The humic substances in the sample volume which neutralized the basic front from pH 13 to a low pH would not sorb on the column and would be lost. Also some humic acids in water, especially in colored waters of high DOC (20 to 75 mg/l), have a tendency to be sensitive to flocculation, even at low concentrations and at a pH of lower than 2. Such flocs of

humic acid do not sorb onto the XAD resins or will partially sorb. Such possible flocculation causes potential problems and should be avoided.

The AG 50W-X8, non-macroporous cation exchange resin used for hydrogen saturating humic substances must be Soxhlet cleaned in the same sequence of solvents as XAD resins. Slurry pack the Soxhlet-cleaned resin into a large glass preparatory column and rinse with distilled water to free the resin of methanol. Hydrogen saturate the resin by slowly passing 15 column volumes of 10% HCl by weight (a volume/volume ratio of 1 to 3 consisting of 1 volume of 36 percent concentrated HCl reagent and 3 volumes of distilled water) through the resin column. The resin should remain in 10 % HCl for an overnight period. Rinse the resin free of excess HCl with distilled water until the conductivity of the effluent is the same as the influent distilled water. Transfer the hydrogen-saturated cation exchange resin to smaller glass precolumns and final saturation columns as specified in the Materials and Methods Section under glassware.

The cation exchange resin has a tendency to bleed slowly when standing in distilled water. It is essential to adequately rinse the cation exchange resin with distilled water before each usage until its hydrogen exchange capacity is exhausted. When the resin becomes sodium-saturated, the resin becomes darker and the color band between sodium-saturated and hydrogen-saturated resin is clearly visible in the column of resin. Upon sodium-saturation of three-fourths of the resin column, remove the resin from the analytical column and resaturate with hydrogen in a preparative column.

Scale, Cost, and Planning for Humic Substances Isolation

The XAD-8 resin procedure for the concentration of humic substances from water can be scaled to accomplish the isolation of mg to 100 g quantities of humic substances. It is only a matter of planning, equipment cost, and time. Time may not be a major factor in some research situations, but because time is cost in most operations, the major factor is the balance of equipment, cost, and time.

For the example given previously, to isolate 400 mg of fulvic acids from a surface water with a DOC of 4.5 mg/l requires the processing of approximately 275 l of water. If the sample were processed on a 2 l column of resin at a k' of 50, 68 l of sample could be processed during each run before elution and regeneration of the resin column. The concentration of the sample could be completed in four sample runs with each run requiring 8 hours (a normal workday) for sample sorption and elution. For completion of the sample in 5 workdays, 55 l of water must be filtered each day (in a 24-hour period because sample filtration can proceed unattended during the night).

Production of 55 l of filtered surface water can usually be accomplished with four filter units of 142-mm size or 1 filter unit of 293-mm size. Equipment cost for pressure filtration equipment, a 2.5 l glass column, four 45 l glass jugs, and pump

accessories for such an experiment is approximately $4,000. The cleaning of 2 l of XAD-8 resin would require a 2-month period prior to the resin use, approximately two man weeks of labor, and an initial investment of $3,000 for a large Soxhlet with a thimble of 2 l capacity. After the one week concentration period on XAD-8, two more man-weeks of time are required for further laboratory processing (humic/fulvic separation, desalting, hydrogen saturation, and freeze-drying) to attain the final hydrogen-saturated, freeze-dried fulvic acid.

In order to concentrate 400 mg of humic acids from the same source of surface water would require 9 weeks of sample concentration time on 2 l, XAD-8 resin columns to process approximately 2250 l of water. To avoid expensive time and labor costs, the concentration process could be scaled-up to be accomplished in one week with the purchase of a 10 l glass resin column, 21 additional small 142-mm plate filter units or 5 additional large 293-mm plate filters, 10 additional 45 l glass jugs, and 2 additional Soxhlet units at a total cost of $30,000. The production of 300 l of filtered surface water per day can be processed on a 9 l resin column during a normal 8-hour workday. Additional sample processing in the laboratory to achieve a final product of 400 mg of humic acids and 3600 mg of fulvic acids, would require an additional 5 man-weeks in the laboratory.

The isolation of 400 mg of fulvic acids from groundwaters and seawaters requires another order of magnitude of time and effort than freshwater stream sampling. Because these waters are relatively sediment-free, the filtration process is not limiting, but the processing of the water sample on the XAD-8 resin columns is time limiting. To process 25,000 l of groundwater or surface water requires approximately 85 workdays (more than 4 months of time) with one 9 l resin column at one run per 8-hour workday. By running two 9 l resin columns simultaneously for 18 hours per day (2 work shifts of 9 hours per shift) the sample concentration time can be reduced to 20 workdays. The capital investment in additional 9 l glass resin columns is minimal, but the time and effort in cleaning an additional 20 l of XAD-8 resin is significant.

From the previous discussion, one can readily understand that to isolate larger gram quantities of humic substances from water or to isolate several 100-mg quantities of humic substances simultaneously from different sources or different depths within a lake requires the appropriate scale factors for equipment, time, and resin cleaning. For the processing of large volumes of surface water, the time limiting factor is usually the rate of water filtration; therefore, the number and size of the plate filter units are critical. Some investigators have used cartridge-depth filters, such as a Balston filter unit, to produce filtered water at a faster rate than can be produced with several membrane plate filter units. The cost of such depth filter units is also much less than for plate filter units. Unfortunately, the filtration process appears to be less efficient in removing suspended-sediment particles and usually higher ash contents are found in humic isolates when using the depth filter cartridges.

Logistics of Water Handling and Processing of XAD-8 Resin Concentrates

The isolation of humic substances from surface waters and groundwaters close to one's own research laboratories usually presents very few problems. Freshwater from the sampling site can be transported daily in stainless-steel or glass containers to the laboratory for processing. When the sampling site is 100 miles or less from the laboratory it is usually more efficient to transport the water to the laboratory than to transport the sampling equipment to the sampling site unless a large, self-contained laboratory trailer is available. When sampling humic substances from surface waters and groundwaters at a great distance from the laboratory, it is usually desirable to transport the sample filtration and resin concentration columns to the sampling site. The combined humic substance concentrates after resin concentration and elution are kept chilled and transported back to the laboratory for further processing.

For concentrating humic substances from seawaters, the author processed the sample on land nearest to the deep ocean sampling site. The resin concentration of humic substances in an onshore laboratory rather than on ship is advocated to decrease the enormous cost of ship time, to preclude the necessity of making all the equipment seaworthy, to avoid seasickness during on-board processing, and to provide for a more normal laboratory working environment. Seawater must be transported from the sampling site to the on-shore field laboratory in glass, glass-lined, or Teflon-coated containers. Transport of seawater in stainless-steel containers is unacceptable due to the corrosive nature of seawater on stainless-steel. The humic substances concentrated after elution from the XAD-8 resin should be transported from the field laboratory to the analytical laboratory for further processing to the point of freeze-drying.

Characterization of the Isolated Humic Substances

Fulvic acids or humic acids are not identified in the classical organic chemical sense; that is, in the same way an unknown compound would be qualitatively identified in qualitative organic chemical analysis. Humic substances are only generally identified by characterization of general properties rather than specifically identified, as are specific organic compounds. Identification by characterization is necessitated because humic substances (either fulvic acids or humic acids) are a group of closely related compounds for which the chemical formula or structure, for all or any one of the components, is not known. The characterization of humic substances may be categorized into three types of analyses: traditional, new, and general characterization methods.

The traditional methods for characterization of humic substances include elemental analysis, titration analysis for carboxyl and phenolic functional groups, E_4/E_6 ratio, ultraviolet spectrographic analysis, infrared analysis, and molecular weight by sephadex chromatography or vapor pressure osmometry. Most of these analyses

indicate that humic substances have a wide range of chemical properties. Newer methods of characterization include solid- and liquid-state ^{13}C NMR spectroscopy, solid- and liquid-state ^{1}H NMR spectroscopy, pyrolysis/mass spectroscopic analysis, δ ^{13}C value, ^{14}C-age, amino acid analysis, saccharide analysis, density, color intensity per unit of carbon, synchronous fluorescence emission, low-angle X-ray scattering, and molecular weight by ultracentrifugation. Several of these methods have been available for sometime, but only recently been applied to humic substances characterization. The general characterizations are for those properties that characterized humic substances on a specific research basis, but are not routinely performed. These include specific metal interactions, differential thermal analysis, various chromatographic separations, chlorine and bromine analysis, methoxyl group content, mass spectroscopy, electron spin resonance (ESR), and many other specific research characterizations.

Even though the traditional characterization methods are generally not very specific for humic substances nor is any characterization a written requirement for characterization of humic substances with the exception that humic substances must be colored; elemental analysis, carboxyl functional group content, and phenolic functional group content are the closest to being generally required characterization parameters. Many of the new characterization methods, such as ^{13}C-NMR and ^{1}H-NMR spectroscopy, are much more definitive for identification of humic substances and are gradually becoming more of a required characterization. For some groups of humic acid scientists, ultraviolet spectroscopy and E_4/E_6 ratios are generally required characterizations, but the relative non-definitiveness of this characterization has been seriously questioned. Because of their definitive nature, the characterization of humic substances by pyrolysis/mass spectroscopy, amino acid analysis, and synchronous fluorescence spectroscopy are expected to become standard characterizations in the near future.

The order in which the various characterizations and the number of characterizations conducted on humic substances is a special consideration in some studies, especially of aquatic humic substances, because of the small amounts of humic substances usually isolated. As presented in the isolation section of this paper, the isolation of large quantities (1 gram or more) of aquatic fulvic or humic acids is not common and is only achieved with considerable effort and expense. With our modern technology, the minimum amount of fulvic or humic acids that can be adequately characterized is 50 mg. The solid state ^{13}C-NMR spectroscopy must be run first because 50 mg is the absolute minimum sample size for high-spectral resolution in a period of 2 to 3 days of spectrometer time. The sample is quantitative recovered and uncontaminated after solid state ^{13}C-NMR analysis. The sample can now be used for various destructive characterization analyses. CHONSP elemental analysis and ash content can be accomplished on 20 mg, titration analysis for carboxyl, phenolic, and ester functional groups on 10 mg, pyrolysis/mass spectroscopy analysis on 1 mg, amino acid analysis on 2 mg, saccharide analysis on 2 mg, infrared analysis on 1 mg, δ ^{13}C value and ^{14}C-age by tandem mass spectroscopy on 2 mg, and combined color intensity per unit of carbon, ultraviolet scan, synchronous fluorescence scan, E_4/E_6 ratio, low angle X-ray scattering, density,

and molecular weight by high-speed ultracentrifugation can all be determined sequentially on the same 7 to 10 mg of sample. A total of 17 characterizations can be accomplished on as little as 50 mg of fulvic or humic acids.

An additional 50 mg or more of humic sample is needed for liquid state ^{13}C-NMR and ^{1}H-NMR spectroscopy. Due to various reagents and solvents used in liquid NMR, it is often difficult to recover the fulvic or humic acids sample in an uncontaminated state.

Summary and Conclusions

Due to low concentrations of humic substances (<2 mg/l) in water and the many interferences from inorganic and other organic constituents, few direct or in situ experiments can be made on humic substances. Also, experiments conducted on the whole DOC of water are usually not representative of humic substances, because humic substances are only approximately one-half of the DOC. For these and other reasons, humic substances must be isolated from water in order to study most aspects of their nature and reactivity. The XAD-8 resin isolation procedure discussed in this paper is one of the best methods of isolating humic substances from water for research purposes.

The success of XAD-8 procedure has been due to many systematic experiments which were conducted to understand the sorptive and desorptive processes on the resin, to incorporate modifications in the procedure which overcome limitations in other isolation procedures for humic substances, the strict adherence to general chemical and chromatographic details, and the incorporation of quantitative chromatographic expressions into the procedure to define the resin sorptive capacity, k', for sorption of humic substances from water on a reproducible basis. The XAD-8 resin isolation method, which has been used successfully for the last few years by a few research groups, is rapidly expanding in usage. A detailed procedure for use of the procedure has been presented in this paper so that it can be used easily and successfully by both experienced chemists and relatively unexperienced scientists in organic chemistry. For the successful use of the method and to obtain uncontaminated humic and fulvic acid isolates, it is imperative that the user give heed to careful and complete cleaning of the resin before usage and to rigorously adhere to all the details and precautions presented concerning the method.

The XAD-8 resin procedure for isolating humic and fulvic acids from water is a time consuming and labor intensive process, but the humic isolates obtained are free from the many limitations which have been encountered in previous efforts to isolate aquatic humic substances. The XAD-8 resin procedure is quantitative for the removal and concentration of humic substances from water. The resin adsorbs 95+ percent of humic substances from water with 100 percent of the adsorbed humic substances being desorbed from the resin. There are no specific interactions between the resin and humic solutes resulting in irreversible sorption of humic substances which could result in low recoveries of humic substances from the resin. Because the isolation

procedure involves pressure filtration, essentially all the clay minerals are removed from the humic substances. The combined treatment of pressure filtration and hydrogen saturation by resin exchange results in humic isolates with low ash contents. The saccharides, low-molecular-weight specific acids, and other hydrophilic low-molecular-weight non-humified compounds in water pass through the resin column and are not adsorbed with the humic substances. Elution of the adsorbed humic substances in dilute base (0.1 M NaOH) results in the elution of only humic substances without admixture with the hydrophobic neutral compounds which remain adsorbed on the XAD-8 resin column. Because the humic isolates obtained by the XAD-8 resin isolation procedure are free from inorganic salts and non-humified specific organic compounds, the author believes and data support the contention that they are truly fulvic and humic acids and not fulvic and humic acid fractions (admixed with saccharides) as in the past.

The sound chemical chromatographic basis of the XAD-8 resin isolation method is a major strength and advantage of the procedure. The use of k' and Equation 2 enables the quantitative and reproducible usage of the method for isolating humic substances from all aqueous environments. The humic isolates from different aquatic environments isolated by this procedure can be compared quantitatively for differences in composition and reactivity. The same criteria used to determine the quantitative and reproducible acceptability of this XAD-8 isolation procedure should be used for any other method developed or existing to isolate humic substances from water.

The XAD-8 resin procedure can be used to isolate mg to 100 g quantities of humic substances from water; it is a matter of scale, time, and equipment cost. Even the isolation of mg quantities of humic substances from water requires a period of 3 to 4 months; a major part of this time is spent cleaning the XAD-8 resin before use. The isolation of 100-g quantities by this method usually requires a minimal equipment investment of $50,000. Even though this method of isolating humic substances from water is time consuming an expensive, it appears to be the method of choice for obtaining quantitative yields of unfractionated humic substances from water.

Humic substances may be characterized in many ways by both traditional and new methods. The new characterizations of ^{1}H-NMR, ^{13}C-NMR, δ ^{13}C content, amino acid analysis, saccharide analysis, and pyrolysis/mass spectroscopy are usually more definitive than older traditional methods. These new methods are rapidly becoming more common and will soon be considered as essential characterizations.

The older traditional characterizations are useful, seldom definitive, and generally augment the newer more definitive characterization methods. The more useful traditional methods include elemental analysis, ash content, titration analysis for carboxyl and phenolic content, functional group analysis by infrared spectroscopy, and molecular weight by various techniques. Some of the traditional characterization methods such as E_4/E_6 ratio, size analysis by sephadex chromatography, and ultraviolet spectroscopy are of limited value.

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Comparison of Aquatic Humic Substances of Different Origin

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Abstract

Fulvic acids isolated by the XAD-method from anaerobic and aerobic landfill leachates are compared with samples isolated from a soil extract and from bog lakes. Elemental composition, functional groups, and spectroscopic and chromatographic data suggest that there is an "ageing" effect from the leachate samples to the soil and brown-water samples.

Introduction

Humic substances (HS) are a class of biogenic, heterogeneous and refractory organic compounds. They constitute the major part of organic carbon in soils and aquatic systems [1,2]. Due to the lack of knowledge about the detailed chemical structure of humic substances, it is difficult to understand their role in the environment [3].

Promising approaches focus on the spatial and temporal variation of samples. Well defined operations are necessary to lay a sound basis for the interpretation of the results [4]. There is no question that the comparison of samples isolated from different sources is the most feasible way to study the genesis of HS.

The goal of the current work was:

1. to isolate the FA fractions from brown-water, soil extracts and landfill leachates,
2. to characterize the samples by chromatographic, spectroscopic and electrochemical methods, and
3. to compare the results and draw some conclusions about the genesis of the HS.

Material and Methods

Origin of samples

The brown-water samples were taken from a bog lake in South Bavaria (Brunnenseemoor; BM 12; Sep 87 [5]) and from a lake in the Black Forest (Hohlohsee; HO 1-2; Aug 88).

Leachates were taken from a landfill near Braunschweig, FRG (BR 5 (O); June 87; BR 6 (A); Aug 87 [6]). BR (A) stands for anaerobic and BR (O) for aerobic water.

Soil extracts were taken from a cultosole (Ah horizon of a cultivated rendzina) of the Munich Gravel Plain, FRG, with 0.1 M pyrophosphate (MUC 2-2; Mar 87; [18]).

Isolation procedure

All samples were isolated according to the XAD-2 method, described elsewhere [8] and outlined in Fig. 1.

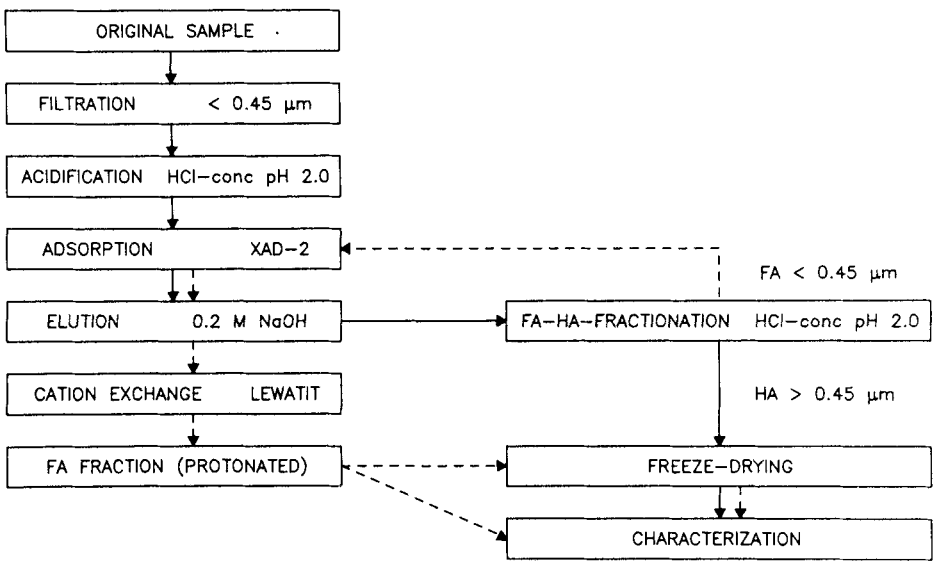

Figure 1 Isolation procedure for the fulvic acid (FA) fraction from aqueous samples.

The original water samples from the bog lakes and the landfill areas were filtered (0.45 µm) before the isolation procedure. The soil sample [18] was extracted at ambient temperature for 70 h with 0.1 M $Na_4P_2O_7$ (5 l per 2 kg soil) at pH 7.0, and the extract was treated like the other aqueous samples.

The freeze dried fulvic acid (FA) fractions were vacuum (10^{-3} mm Hg) dried at 70°C for elemental analysis and IR-measurement.

Aqueous solutions of defined mass concentration were prepared with double distilled water for all other investigations.

Analytical determinations

The determination of C, H, O, N and S was done as previously described [7]. Dissolved organic carbon (DOC) concentrations were determined in membrane (0.45 µm) filtered solutions using a UV/DOC analyzer MAI 3 (MAIHAK).

The proton capacity was determined by titration of deaerated solutions of pH from 3.0 to 11.0, using an autotitrator (DL 25; METTLER) connected to a printer plotter (FX 800; EPSON) [7].

The complexation capacity (CC) for Cu(II) was measured by differential pulse polarography (DPP) as previously described [8] and by the fluorescence quench method [9].

The molecular weight distribution was determined by gel permeation chromatography according to the method described by Fuchs [10]; a detailed protocol has been presented by Weis et al. [7]. Briefly, 1 ml of the concentrated sample (ca. 150 mg DOC/l) was applied to the TSK column and chromatographed with 0.15 M phosphate (pH 7.0) as mobile phase.

IR-spectra were measured using the KBr technique with a FT-IR spectrophotometer FTS 50 (DIGILAB).

The spectral absorbance at 254 nm and 436 nm was measured with a spectrophotometer (LAMDA 5, PERKIN ELMER and PYE UNICAM SP 8-100, PHILIPS) using 1 cm and 5 cm quartz cells. The pH of the solution was 11.0, and double distilled water was used as reference.

Results

The elemental composition of the FA samples is given in Table 1.

It is obvious that the FA fraction from the anaerobic leachate BR 6 (A) has by far the highest content of C, H and S. The C/H ratio is small and the C/O ratio is high compared to the other samples. There is not much difference in the other values, which are in good agreement with literature data [11]. It is interesting to note, that the sample from the anaerobic leachate (BR6 (A)) fits better into the group of humic acids (HA) than into the family of FAs [12].

Table 1 Elemental composition (%, dry weight) and elemental ratios of the isolated FA-fractions.

	Bog lake **(BM 12)**	Brown water **(HO 1-2)**	Leachate **(BR 5 (O))**	Leachate **(BR 6 (A))**	Soil extract **(MUC 2-2)**
C	53.2	51.6	51.9	57.9	49.4
H	3.4	3.9	4.3	5.9	3.8
N	2.2	3.2	3.7	2.3	3.9
O	40.2	37.2	36.0	30.4	40.5
S	0.9	0.9	1.8	2.3	0.9
Ash	0.1	3.2	2.3	1.2	1.5
C/H	1.3	1.1	1.0	0.8	1.1
C/O	1.8	1.8	1.9	2.5	1.6

The spectroscopic behaviour in the UV and visible ranges leads to further differentiation (Table 2). The FAs from the soil and the bog lakes absorb similarly, however, in the case of the FAs from the leachates, absorption is less intense. The anaerobic sample has by far the weakest spectral absorbance. In addition, the absorption at 254 nm for the leachate FAs is relatively strong compared to the yellow colour at 436 nm.

Table 2 Specific spectral absorbance at λ= 254 nm (A 254) and at λ= 436 nm (A 436) of dissolved FAs (l/(m x mg DOC); pH 11.0).

	Bog lake (BM 12)	Brown water (HO 1-2)	Leachate (BR 5 (0))	Leachate (BR 6 (A))	Soil extract (MUC 2-2)
A 254	6.5	5.7	3.4	1.0	5.3
A 436	0.62	0.64	0.26	0.09	0.60
A 254/A 436	10.3	8.9	12.9	11.2	8.6

Ligand functional groups contribute to the typical properties of FAs. Proton capacities and complexation capacities are well suited for comparison, even though the values are operationally defined. Table 3 shows that all FA samples have a total proton capacity (Σ) of around 15 µmol/mg DOC, with acidic groups (pH $\leq$ 7) contributing about 11 µmol/mg DOC and less acidic ones (pH > 7) contributing about 4 µmol/mg DOC.

Table 3 Proton capacities (µmol/mg DOC) of isolated FAs.

	BM 12	HO 1-2	BR 5 (0)	BR 6 (A)	MUC 2-2
≤ pH7	11.2	9.8	10.7	11.5	12.4
> pH7	5.8	2.0	3.0	3.5	4.2
Σ	17.0	11.8	13.7	15.0	16.6
≤ / > pH7	1.9	4.9	3.6	3.3	3.0

The anaerobic sample, in particular, has a very small complexation capacity (Table 4). The polarographically determined values (POL) are significantly higher than the ones measured by the fluorescence quench method (FLQ). Although this frequently observed effect [9] needs further investigation, it is attractive to assume that, in the case of polarography, adsorption effects add to the complexation reaction, whereas the fluorescent regions might not all be quenched by complexed paramagnetic metal ions.

Table 4 Cu(II)-complexation capacities (µmol/mg DOC) of isolated FAs.

	BM 12	HO 1-2	BR 5 (0)	BR 6 (A)	MUC 2-2
POL	2.7	2.3	1.8	0.8	3.3
FLQ	1.6	1.2	1.2	0.2	2.6

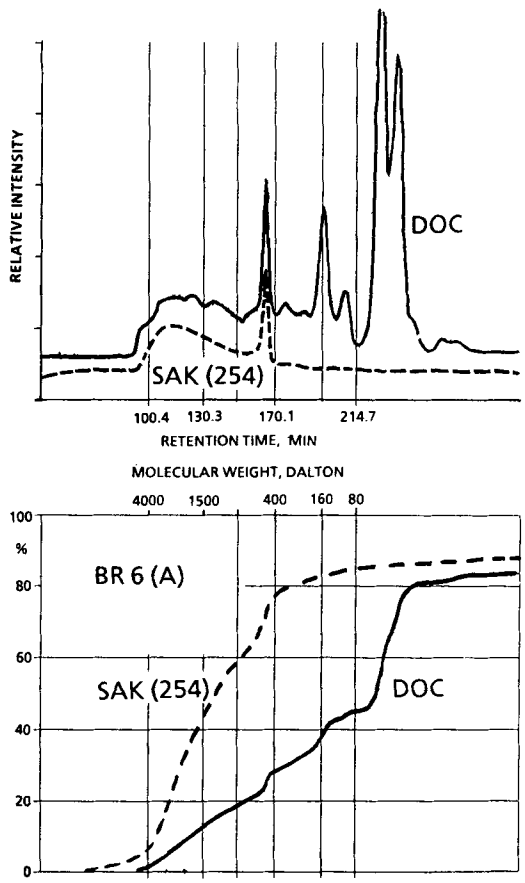

Figure 2 Gel permeation chromatogram and molecular weight distribution for BR 6 (A) FA; correction for retention time UV-DOC = 300 s.

The molecular structure of humic substances cannot be described in a non-degradative way, and it is therefore desirable to learn more about the general physical and chemical characteristics of the individual samples. Liquid chromatograms are well suited to give information on polarity, molecular weight distribution, etc. [13], even though there can be serious drawbacks [14]. Fig. 2-5 show the chromatograms gained by gel permeation. The chromatograms were run with simultaneous detection of the DOC and the spectral absorbance at 254 nm (SAK 254). The areas under both chromatograms were integrated to give the molecular weight distribution, shown below the chromatograms.

It has to be kept in mind, that the molecular weight numbers are based on the calibration with various compounds listed in the experimental section. The lack of identity with FAs is unsatisfactory, therefore the molecular weight distribution can only be seen as an estimate and is referred to as an apparent molecular weight distribution.

The sample from the anaerobic leachate (Fig. 2) shows a relatively small amount of DOC in the higher molecular weight range. The difference between the DOC line and the spectroscopic line is striking, especially in comparison to the other samples. The aerobic

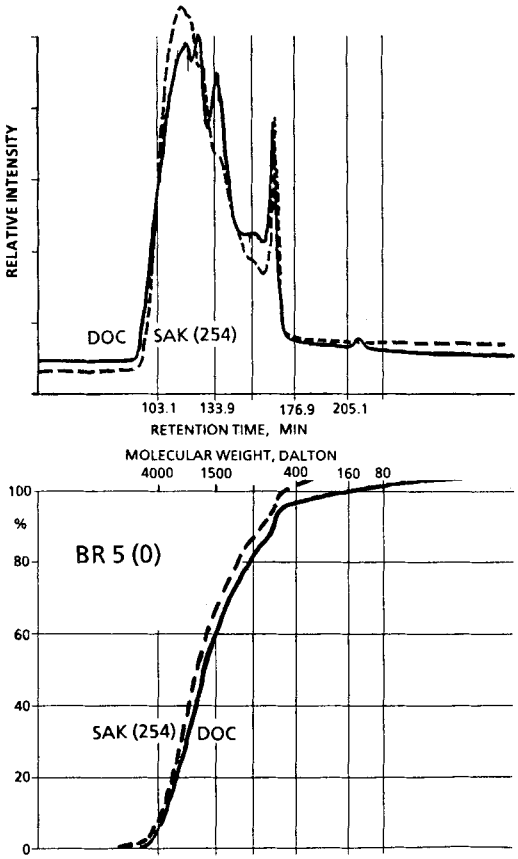

Figure 3 Gel permeation chromatogram and molecular weight distribution for BR 5 (O) FA; correction for retention time UV-DOC = 300 s.

leachate sample (Fig. 3) has more than 50% of the matter in the range of a few thousand Dalton. Characteristic peaks occur around 1500 and around 600 Dalton, although the peak at 1500 Dalton has a relatively low UV absorbance. The FA sample from the soil (Fig. 4) contains the highest molecular weight substances, and nearly 80% of the sample has a nominal weight higher than 1500 Dalton. A smaller fraction appears at about 400 Dalton. The chromatogram and distribution for the FAs from the brown water (Fig. 5) are similar to the chromatogram for the FAs from the bog lake. They are least structured and closest to a normal distribution with a mean between 2500 and 1500 Dalton. The similarity to the main fractions of the sample from the aerobic seepage water is obvious. Most of the soil FAs show higher and most of the anaerobic leachate FAs show lower molecular weights. The high molecular weight fraction of BR 6 (A) behaves chromatographically similar to the brown water FAs, indicating that a general maturing of aquatic FAs can be derived.

A survey of the dominant bonds and functional groups of FAs can be obtained with infrared spectra (IR). Assignments for the absorption bands of humic substances can be found in the literature [1,15,16]. Absorption bands of defined substances can be compa-

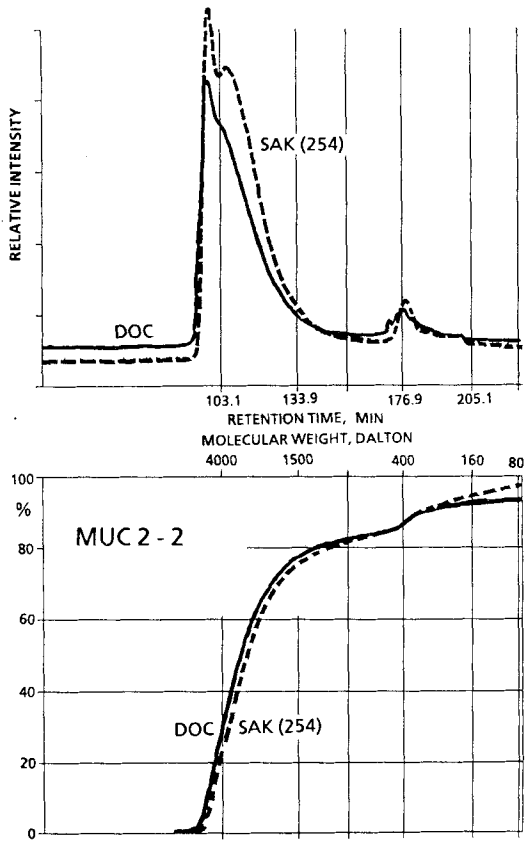

Figure 4 Gel permeation chromatogram and molecular weight distribution for MUC 2-2 FA; correction for retention time UV-DOC = 300 s.

red with the data collected by Bellamy [17]. Especially the Fourier transformed (FT) data reveal typical bands for the protonated samples (Fig. 6). The broad band around 3400 cm^{-1} and 3200 cm^{-1} are due to O-H stretching bonds of phenolic structures or alcohols. There is strong evidence for intermolecular and intramolecular hydrogen bonds. The band at 3530 cm^{-1} of the soil derived sample can be explained by single bridged O-H stretching bonds. The relatively sharp bands at 2967 and 2940 cm^{-1} are due to aliphatic C-H stretching bands. They are most obvious in the anaerobic leachate FAs and fit well to low C/H ratio. The shoulder at 2600 cm^{-1} can be assigned to the hydrogen bonded O-H stretching vibration of carboxylic acid groups. The dominant band at 1720 cm^{-1} is very likely caused by the C=O stretch vibration of acids, aldehyds and ketones. Typical for the brown water FAs is the second band at 1640 cm^{-1}, which is due to C=C stretching vibration of unsaturated and aromatic structures. Conjugation of C=O with C=C can also contribute to the absorption. The strong bands in the HO 1-2 sample and in the BM 12 sample (not shown) parallel the high spectral absorbance at 254 nm (A 254, Table 2). It is also tempting to assume that the IR band is connected with keto-enol-tautomerism, which leads to stable metal complexes by chelation. A high complexation capacity for Cu(II) (Table 4) favours this hypothesis. The O-H bending vibration of alcohols and carboxylic acids at 1440 cm^{-1} is also strongest in the brown water FAs, whereas the

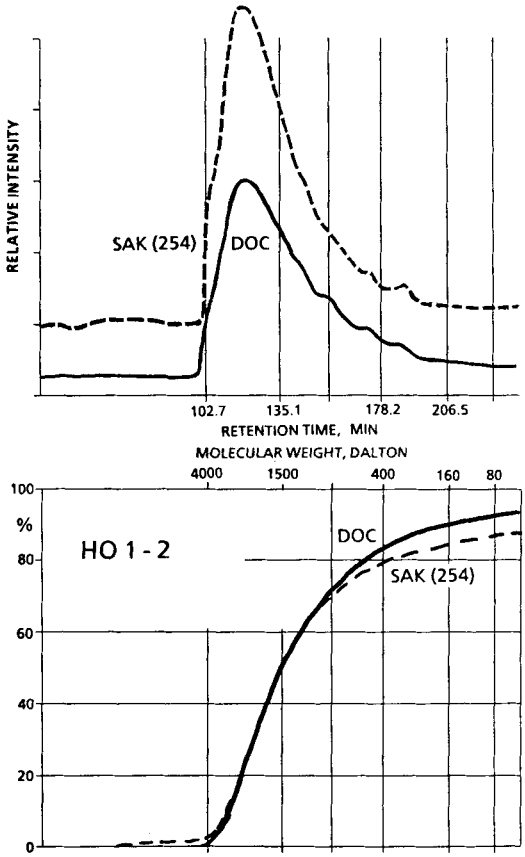

Figure 5 Gel permeation chromatogram and molecular weight distribution for HO 1-2 FA; correction for retention time UV-DOC = 300 s.

combination of the O-H deformation band and the C-O stretching band of ethers, esters and phenols at 1215 cm^{-1} is dominant in all other samples. Whether the relative intensity of the band around 1440 cm^{-1} and 1215 cm^{-1} are typical for the genesis of FAs or not must be further investigated.

Conclusions

Meaningful characterization of humic substances must include a clean definition of the origin and clearly described isolation procedures. On that basis samples from different origins can be compared with one another. A multiple method approach is suited to characterize the samples.

The results for FAs isolated from anaerobic and aerobic landfill leachates, from a soil extract and from bog lakes, lead to the following conclusions:

- The sample from the anaerobic leachate differs most from all other samples. The C/H ratio is low and the C/O ratio is high.

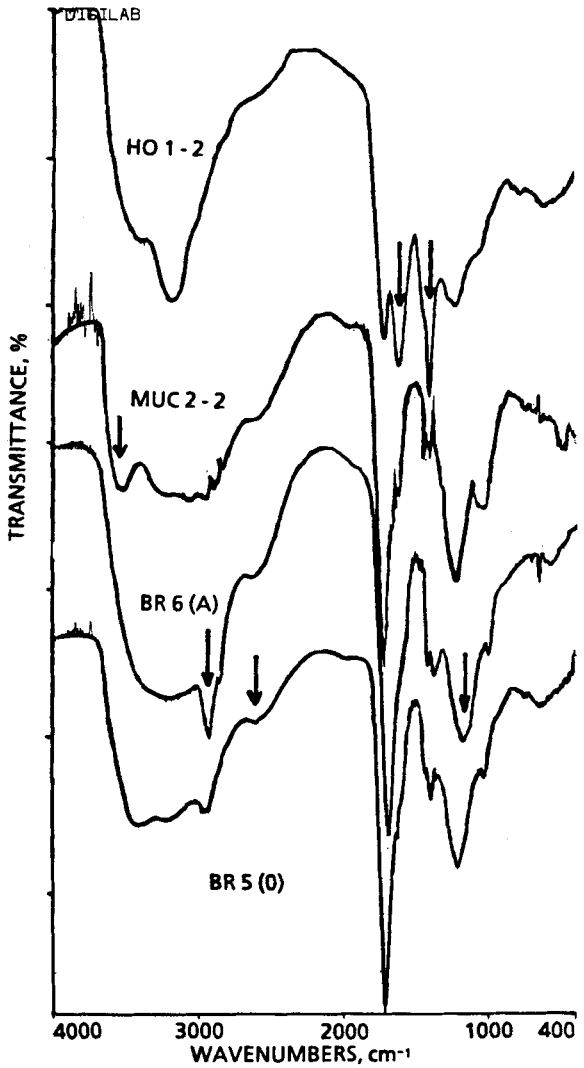

Figure 6 FT-IR spectra for the isolated FAs (KBr disk; resolution 2.0 cm $^{-1}$; 64 (MUC) and 256 (all others) scans).

- A relatively weak spectral absorbance and the low amount of functional groups suggest that the anaerobic FAs are fairly young.
- Ageing leads to products more like the aerobic FAs or the ones isolated from soil and brown-water.
- A large portion of the substances have an apparent molecular weight of about 2000 Dalton, and there is a larger amount of lower molecular weight material in case of the anaerobic FAs.
- The dominance of the band at 1640 cm^{-1} in the IR spectra seems to be typical for FAs from aqueous systems stabilised by ageing.

Acknowledgements

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Structural Features of Aquatic Fulvic Acids by Analytical and Preparative HPLC Followed by Spectroscopic Characterization

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Abstract

Analytical and preparative reversed-phase liquid chromatography were used to separate constituents of Suwannee River aquatic fulvic acid. Photodiode array UV-Vis, and fluorescence detectors were used to monitor the HPLC separation. Preparative FA fractions were subjected to ^{1}H and ^{13}C solid state NMR, FT-IR, and ESR. The preparative and analytical RP-HPLC separations have shown that the FA macromolecule consists of at least two polymeric fractions, each of which can further be separated into individual constituents by analytical RP-HPLC. Vanillic acid structures were identified in the analytical chromatograms of the total FA sample, but not in the fractions. The first fraction included the hydrophilic constituents of FA and represented about 40 percent of the total sample. It could be resolved into six constituents by analytical RP-HPLC. Their t_R and UV-Vis scans were characteristic of carboxylic acids in rigid structures which exhibit strong hydrogen bonding. The second fraction included the hydrophobic constituents and represented about 30 percent of the total sample. It could be resolved into twelve constituents. Repeated structurally related units were separated and their UV-Vis scans were characteristic of conjugated ketone and phenolic structures. Free radical structures were present in both hydrophilic and hydrophobic fractions.

Introduction

The structures of many naturally occurring compounds have recently been elucidated through the combined use of preparative HPLC followed by NMR, FT-IR or mass spectrometry [1-4]. For example, carotenoid pigments have been sucessfully separated by HPLC. The ^{1}H and ^{13}C spectra of as little as 2 mg of a pure compound were interpretable [5]. In the case of FA, the complexity and heterogeneity of the macromolecules, even after elaborate isolation and purification, continue to limit the abilities to develop detailed structural information. Several recent ^{1}H- and ^{13}C-NMR and FT-IR studies [6-9] have provided useful information but so far, no real breakthrough has been developed.

Recent ^{13}C NMR studies on FA and humic acids were centered around the aromaticity and the ratio of aliphatic to aromatic carbons. New techniques such as ^{13}C - ^{1}H

dipolar dephasing [10], spin-echo and broad band decoupled ^{13}C CP/MAS [11] were utilized in humic substances research. A recent spin counting experiment [12], indicated that 97% of the carbons in Suwannee River fulvic acid, (SR-FA) can be detected by ^{13}C CP/MAS. Hydroxyl and aromatic ketone groups in FA and HA were examined with ^{13}C NMR for chemically derivatized samples [13,14]. With CH_3I/NaI permethylation, the ratio of carboxylic acid hydroxyl to total hydroxyl content could be determined. Products of side reactions by derivatization were identified. It was estimated that about one ketone group per monocyclic aromatic ring exists in both humic and fulvic acids. The pH effect on the dissociation of carboxyl and phenolic groups of HA were observed by ^{13}C-NMR spectroscopy [15].

It seems that the inability to develop detailed structural information on FA can be attributed to two factors. One is the inability to unfold or fractionate the constituents of FA into small molecular constituents. The other is the lack of detection of orderly repeated structural units in the FA macrostructure. Until now the polymeric nature of FA has not been unequivocally verified [6].

This paper presents the results of an investigation of the structural features of aquatic fulvic acids and HPLC fractions by solid state ^1H and ^{13}C NMR, FT-IR and ESR. Detailed information on the analytical and preparative separations have recently been published [16, 17]. The overall approach in this research was to utilize purified FA as starting material, to carefully evaluate the preparative and analytical separations, and to utilize combined techniques to develop structural information.

Materials and Methods

Samples

Total samples included reference and standard Suwannee River fulvic acids, purchased from the International Humic Substances Society [18]. Preparative HPLC fractions included the hydrophilic (A-1) and the hydrophobic (B-1) fractions, separated by preparative RP-HPLC as described [16]. Table 1 shows a summary of the mobile phases, columns and gradient programs.

Instrumentation

A Waters model 201 HPLC, equipped with a fixed UV detector (Beckman Model 160) and a fluorescence detector (Schoeffel Model 970), was used in the first preparative experiment. A Hewlett Packard HPLC model 1090 with a model DR-5 pump and a UV-Vis photodiode array Detector (DAD) was used in all other experiments.

NMR experiments included ^{13}C cross polarization magic angle spinning (CP/MAS) and ^1H combined rotation and multiple pulse spectroscopy (CRAMPS) on total reference fulvic acids FA-T and on the two HPLC factions. ^{13}C NMR spectra were obtained with a Nicolet NT-150 spectrometer at 37.7 MHz using a home built CP/MAS modification including the probe. The cross polarization contact time was 1 ms and the pulse repetition time was 1 s. The ^1H irradiation field was 11G and 1K data points were zero filled to 2K points in the spectra. Chemical shifts were measured with respect to tetramethylsilane via hexamethylbenzene as a secondary substitution reference (aromatic peak at 132.3 ppm).

Table 1 Continuous gradient programs and types of columns and samples used.

Gradient Program	Mobile Phase	pH at 25°C	Gradient	%A	%B	Column Types	Samples
I	A: H_2O + 0.01% AcH B: CH_3CN	4.0	t_0 min t_2 min t_{16} min t_{20} min	99 70 15 15	1 30 85 85	-Novapak C 18 5 µm 100 mm L x 3.9 mm ID Flow 0.5 ml/min -Hypersil ODS C18 200 mm x 2.1 mm ID Flow 0.3 ml/min	SR-FA, HPLC fractions, model compounds SR-FA, HPLC fractions
II	A: H_2O (He Purged) B: CH_3 CN	7.0	t0 min t_2 min t_{16} min t_{20} min	99 70 15 15	1 30 85 85	-Novapak C18 5 µm 100 mm L x 3.9 m ID Flow 0.5 ml/min -Hypersil ODS C18 200 mm x 2.1 mm ID Flow 0.3 ml/min	SR-FA, HPLC Fractions, Model Compounds SR-FA, HPLC Fractions
III	A: H_2O (He Purged) B: CH_3OH	7	t_0 min t_{30} min t_{35} min t_{45} min t_{50} min	99 99 15 15 15	1 1 85 85 85	-Custom made Novapak C18 4 µm 300 mm L x 7.8 mm ID Flow 1.5 ml/min -Novapak C18 5 µm 150 mm L x 4 mm ID	SR-FA Second Preparative Experiment SR-FA, HPLC Fractions

Usually 13000-50000 scans were accumulated. Bullet-shaped spinners were used with a sample volume of 0.4 ml and were spun at about 3.8 kHz. The ^1H CRAMPS spectra were recorded on a modified NT-200 at a proton Lermor frequency of 187 MHz using the BR-24 sequence. The Br-24 cycle time was 108 ms corresponding to $\tau = 3.0$ µs. The RF field strength, V 1H was 52.5 G. Chemical shifts are reported relative to the proton resonance of TMS and are accurate to $\pm$ 0.3 ppm. The FT-IR instrument was a Nicolet Model 60 SAB equipped with Spectra-Tech DRIFTS attachment. The operating conditions were: 265 scans, KCl sample background, resolution 1 cm^{-1}, DGTS detector. The ESR spectrometer was a Varian model 4502. ESR spectra were recorded on the solid samples in a quartz tube of 14.6 cm (L) x 4 mm (ID) at room temperature (24 $\pm$ 1°C). The measurements were made under the following conditions: Frequency, 9.5 GHz; modulation frequency, 100 kilocycle (Kc); magnetic field setting, 3,350 to 3,370 gauss; standard for solid state, 2,2-diphenyl-1-picrylhydrazyl (DPPH) diluted with KC1; and standard for liquid phase, DPPH in benzene.

Table 2 Preparative experiments 1 and 2. Percent recoveries of fractions.

	Initial total wgt in mg	Wgt of A-1 fraction mg	Wgt of B-1 fraction mg	% Total recovery A + B	% of A-1 recovery from total FA	% of B-1 recovery from total FA
Preparative Experiment 1						
Combined batches (1-5)	50	19.0	11.6	61.2	38.0	23.2
Combined batches (6-8)	30	11.20	9.8	70	37.3	32.7
Total from experiment 1	80	30.20	21.4	64.5	37.8	26.8
Preparative Experiment 2						
Combined batches (1-2)	20	7.91	6.51	72.1	39.6	32.6
Combined batches (3-4)	20	9.29	5.98	76.4	46	29.9
Total from experiment 2	40	17.20	12.49	74.2	43.0	31.2
Mean recoveries experiments 1 and 2	-	-	-	69.4	40.4	29

Results and Discussions

Preparative and Analytical HPLC

The total recoveries of FA and the contribution of each fraction are shown in Table 2.

Gradient Program I - Novapak Column

Fig. 1 shows the analytical chromatogram and the UV-Vis scans of a total SR-FA sample dissolved in a pH 7 buffer. Six peaks are resolved. The scans of the first two peaks are featureless and showed notable decrease of absorbance with increase of wavelength. Peak 3 is well defined and symmetrical and its scan is featureless and closely resembles some of the published UV-Vis spectra of aquatic FA [19]. Peak no. 4 is a stronger and broader one. However its spectrum is very similar to peak 3. This is rather interesting in view of the ideal peak shape and symmetry of peak no. 3. While both peaks, no. 5 and 6, differ by one minute in t_R, the UV-Vis scans are almost the same. Both spectra show the distinct absorption lines at 260 and 290 nm, characteristic of vanillic acid structures. The t_R and uv scans of standard vanillic acid are similar to those of peak no. 5. This may be one of the first detections of vanillic acid structural units in FA, via HPLC analysis. Additional weak UV and visible absorption lines were detected in the scans indicating that the VA structures are not present in the pure acid form. The analogy between the UV-Vis scans of vanillic acid (VA) and those detected in aqueous solutions of FA leaves little doubt regarding the presence of vanillic acid structural units in the FA macro-molecule. As discussed below, it is interesting to note that the vanillic acid structures are not detected in the chromatograms of either fraction A-1 or B-1. A possible explanation is that vanillic acids are only intermediates formed by the slow degradation from lignin precursors. Once the macromolecule is fractured during the HPLC seperation, VA may undergo further degradation to simpler carboxylic or phenolic compounds.

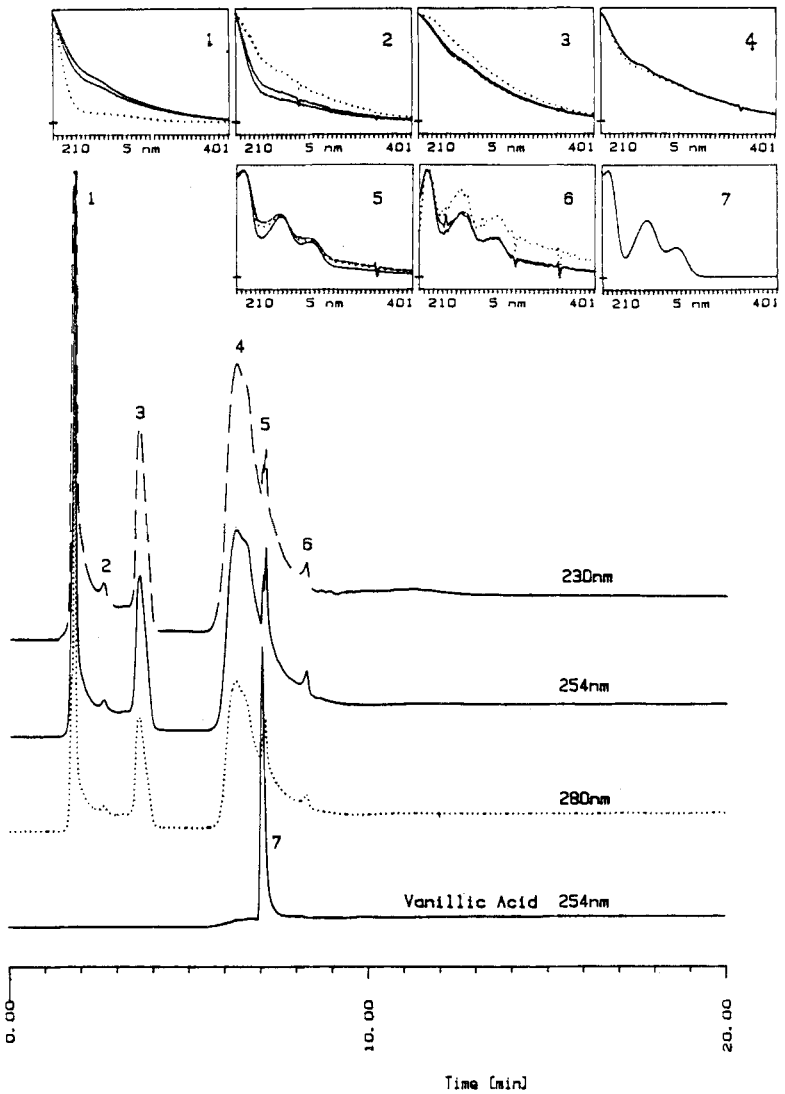

Figure 1 Chromatogram and UV-Vis scans of 50 µl 0.02 % SR-FA and 50 µl 0.02 % vanillic acid in pH 7 buffer. Gradient program I.

Gradient Program I - Hypersil C_{18} Column

Fig. 2 shows the chromatogram and UV-Vis spectra of 50 µl of an aqueous 0.02% SR-FA solution and the hydrophilic fraction A-1. The UV-Vis scans of the peaks 1-3 are all featureless and similar to the scans of the early eluting peaks with the Novapak column. Peaks 4 and 5 are also featureless, but show some absorption lines in the visible region indicating the presence of chromophores and auxochromes. This scan represents the predominant polymeric structure in FA.

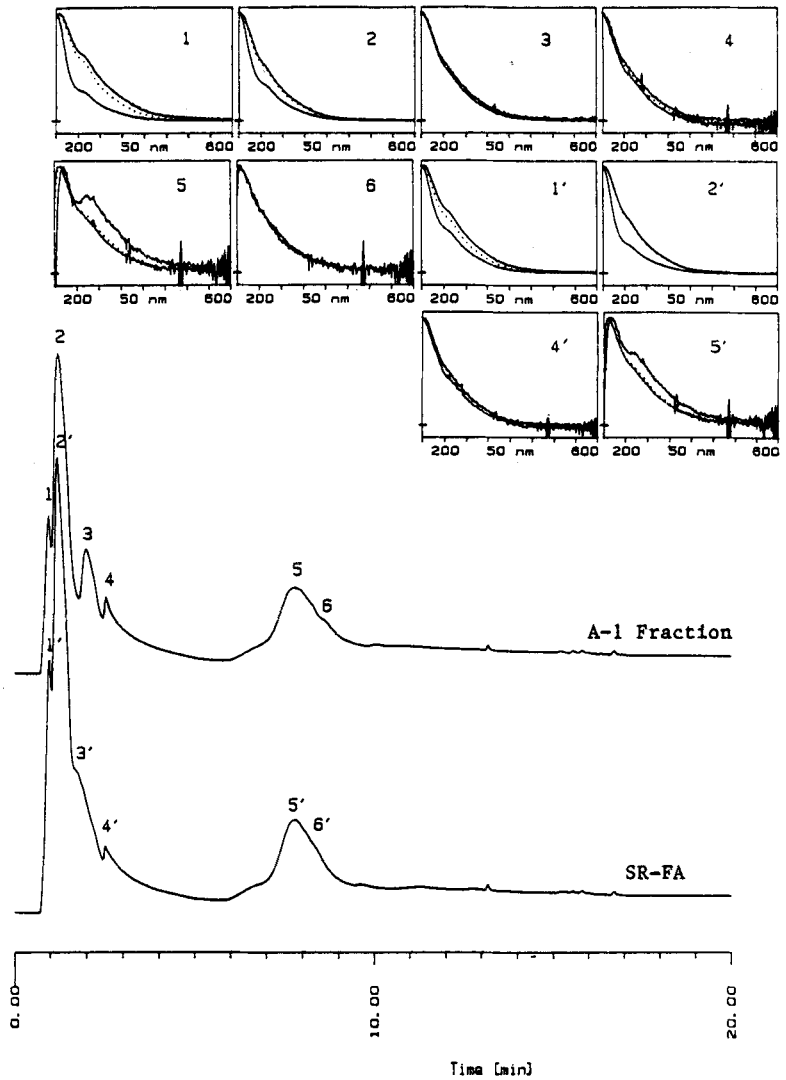

Figure 2 Chromatogram and UV-Vis scans of 50 µl 0.02 % SR-FA and 50 µl 0.02 % A-1 fraction dissolved in MilliQ water. Gradient program II. Hypersil ODS column.

Fig. 3 shows the chromatogram and scans of the hydrophobic fraction B-1. The chromatogram was monitored at λ = 230 nm. The scans of the early eluting peaks in the first two minutes are similar to those present in the total sample and hydrophilic fractions. Peaks 2-12 were eluted between 6 and 18 minutes with only peak 2 indicating a broad signal with a scan characteristic of the predominant colored fragment of FA. Peaks 3, 5, 6, 7, and 8 showed distinct absorption line at λ = 226 nm and a weak line at 265 nm. The similarities and intensities of these peaks suggest the presence of repeated structural units in the FA macromolecule. It should be remembered that these peaks occurred between t_R

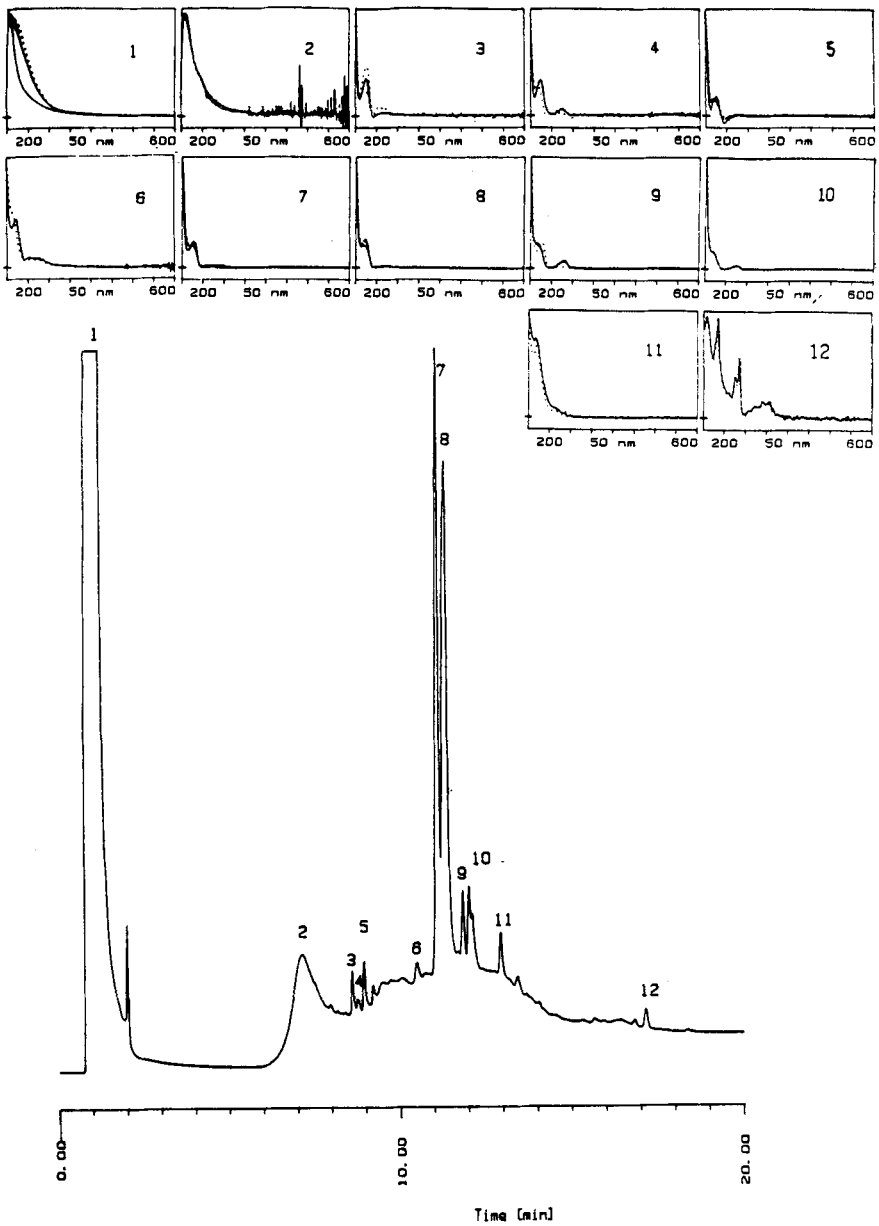

Figure 3 Chromatogram and UV-Vis scans of 50 µl 0.02 % aqueous hydrophobic fraction B-1 dissolved in methanol. Preparative experiment 2. Gradient program I. Hypersil ODS Column.

8.62 and 11.42 minutes i.e. within 2.8 minutes in the hydrophobic region. It is suspected that these peaks correspond to conjugated ketone structures. Scans of peaks 4, 9 and 10 are all similar and contain a defined additional absorption line at 275 nm. Such UV

spectra are characteristic of phenolic compounds. In peak 11 the 275 nm line is barely detectable but a well defined line at 221 nm is noticeable. Peak 12 occurred at 17.2 minutes and t_R show several absorption lines at 209, 235, 260, 275, and 285 nm. These lines are characteristic to polynuclear aromatic structures. The chromatogram of Fraction B-1 on the Hypersil column represents one of the most successful chromatographic separations of FA. Their UV-Vis scans provide the first evidences of the presence of structurally similar units in FA. The effect of pH on the B-1 chromatogram was evaluated by injecting the same sample using mobile phase II at pH 7. The chromatogram showed essentially the same resolution and UV-Vis scans. The effect of storage of the B-1 solution was evaluated by injecting the same sample solution under the same conditions, after storage at 4°C for 5 months. The chromatogram showed essentially the same resolution and UV-Vis scans. The only detecable difference was the increase of the magnitude of the phenolic peaks and the decrease in the magnitude of the conjugated ketone peaks.

Solid State Proton CRAMPS NMR

Fig. 4 shows a comparative ^1H CRAMPS Spectrum of total FA and the two HPLC fractions. Notable improvement is shown in the spectrum of the B-1 fraction. The spectra of Total FA and A-1 fraction show broad overlapping lines, representing resonances at δ = 0.5, 1.8, 3.00, 7.00 and 10.5 ppm. Protons can be assigned to the following regions: i) the alkane protons between 1-2 ppm, ii) the hydroxyl protons between 3-4 ppm; iii) the aromatic protons between 7-9 ppm and iv) the acidic protons between 10-12 ppm. Detection of the acidic protons in a 24 h air dried sample verifies the assignment of acidic protons. It is interesting to note that the data on aromaticity of FA by ^{13}C and ^1H NMR represented 14.1 and 13.5%, respectively. These results are in good agreement with published data on SR-FA.

The ^1H CRAMPS spectrum of B-1 shows well resolved resonance lines corresponding to the alkyl, O-alkyl and aromatic protons. The broad signal centered at about δ = 2.5 ppm corresponds to labile protons. Most of the intensity in the FA-B spectrum is in the aliphatic region and is remarkably well resolved. The most upfield line is at δ = 0.25 ppm. The line width in Fig. 4 ranges from 2-4 ppm which is considered excellent for a solid polymeric materials such as FA. Considering the hydrophobic nature of the B-1 fraction, the proton distribution reflects an essentially aliphatic structure where at least 50 percent of the protons are labile and correspond to orderly arranged structures. Combined with evidence from the UV-Vis scans and the FT-IR, conjugated ketones are the major constituents of this fraction. The percentage of aromatic protons are 5.6, which is consistent with the information derived from the HPLC results.

Solid State CP/MAS - ^{13}C NMR

Fig. 5 shows the ^{13}C spectra of total SR-FA and the hydrophobic fraction B-1. The major ^{13}C resonance lines in the total sample are very comparable to those reported in the literature on SR-FA. It is interesting to note that our calculated percentages of aromatic and carboxylic carbons are 14 and 18 percent, respectively. These results are in good agreement with the data derived from the ^1H-CRAMPS as well as the published data on SR-FA [13].

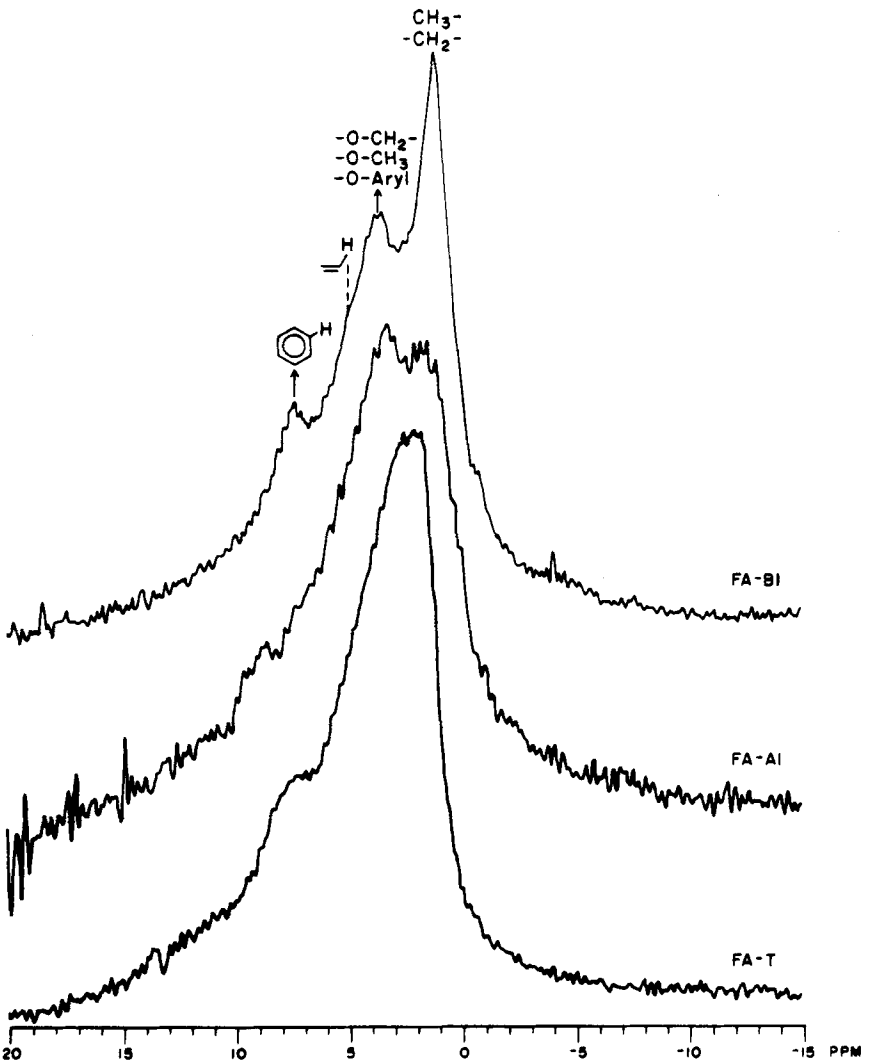

Figure 4 Solid state ^{1}H CRAMPS of fulvic acids and two HPLC fractions.

The CP/MAS ^{13}C spectrum of the hydrophilic fraction A-1 did not show any measurable signal. This fraction weighed ~ 30 mg and was expected to produce better signals than the B-1 fraction. The inability to obtain a good ^{13}C spectrum on this sample may be due to the presence of trace amounts of metallic paramagnetic contaminants. As discussed in the ESR section, this sample shows several ESR absorption lines with coupling constants in the range of 60-70 gauss.

The hydrophobic fraction B-1 weighed 21 mg and it was suspected that a useful ^{13}C spectrum could be obtained from such as small amount. Indeed it was difficult to obtain a spectrum of this sample. As shown in Fig. 5, the ^{13}C CP/MAS of the FA-B-1 is very

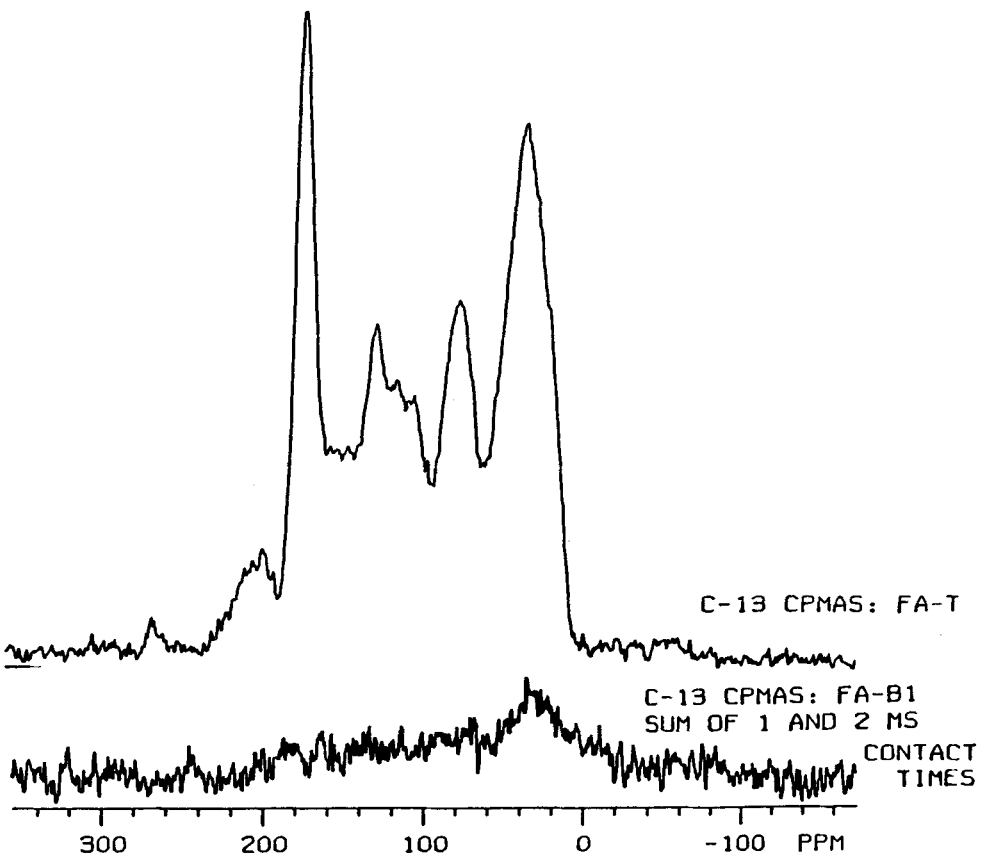

Figure 5 Solid state ^{13}C CP/MAS spectrum of the FA-T and B-1 fraction.

weak. The only notable feature of the specturm is the predominance of aliphatic structures.

Fourier Transform IR

Fig. 6 shows the FT-IR spectra of total SR-FA and fractions A-1 and B-1. Four IR absorption regions are discussed.

Absorption in the 3400 cm^{-1} region is due to the OH stretching and its broadness is usually attributed to hydrogen bonding. The feature in this region is broad and extends between 3300-3700 cm^{-1} indicating the presence of OH groups vibrating over a wide range of energies. This range may cover various types of OH stretching. For example, aliphatic alcohol absorb at 3640-3600 cm^{-1}, phenolic OH at 3612-3593 cm^{-1}, peroxide OH at 3550 cm^{-1}, polymeric hydrogen bonded OH at 3400-3200 cm^{-1} and chelated OH at 3200-2500 cm^{-1}. The flat broad band at 2600 cm^{-1} can be attributed to OH stretching vibrations of the carboxyl groups.

Examination of Fig. 6 shows that the total FA and the hydrophilic fraction, have similar bands in this region. This is expected since both samples contain substantial OH groups from carboxylic acids and phenols. In the hydrophobic fraction B-1 this region is significantly reduced, and extends over a narrower range between 3600-3100 cm^{-1}. It seems better resolved from the next region which extends between 3000-2800 cm^{-1}. Since this fraction contains only hydrophobic constituents, polymeric hydrogen bonded OH may be the major contributor to the absorption in this region.

Absorption in the 3000-2750 cm^{-1} region is caused by aliphatic CH stretch and is expected to be enhanced by methylation resulting from introduction of CH_3 group. The aromatic stretching mode occurs at 3055 cm^{-1} and the aliphatic CH stretch frequencies at 2970 and 2870 cm^{-1} (CH_3) and 2920 and 2850 (CH_2).

Fig. 6 shows that the total sample has two poorly resolved bands in this region at 2960 and 2920 cm^{-1}, which can be assigned to the symmetric and antisymmetric stretching vibrations of aliphatic CH bands of CH_3 or CH_2 groups. Methylation would increase absorption in this region. In the spectrum of the hydrophilic fraction, this region is slightly more resolved than in the total sample. In the spectrum of the hydrophobic fraction, this regions is well enhanced and is resolved into four distinct but overlapping bands at 3010, 2950, 2880 and 2850 cm^{-1}. The enhancement of this region is due to marked decrease in the OH stretching region and the mild methylation due to the use of 85% CH_3OH as a mobile phase, during the HPLC separation. It is interesting to note the appearance of a shoulder at 3010 cm^{-1} indicating some aromatic C-H stretch that was masked in the total sample and hydrophilic fraction. A distinct but small shoulder appears at 2720 cm^{-1} which may indicate C-H of an aldehyde.

Within the 2700-1950 cm^{-1}region the broad band at 2600 cm^{-1} in the total sample can be attributed to the broad hydrogen bonded COOH. The band is less pronounced in the hydrophilic fraction. The total sample shows a minor shoulder at 2380 cm^{-1}. The hydrophilic fraction shows a well defined band at 2350 cm^{-1}. The hydrophobic fraction has two absorption bands in this region, one is centered at 2550 cm^{-1} and the other is centered at 2120 cm^{-1}. Absorption in this region corresponds to triple bond vibrations.

The major part of absorbances in the 1720-1450 cm^{-1} region is caused by C=O stretching of COOH group which absorb at 1725 cm^{-1}. This band often shifts to a slightly higher frequency upon methylation. This is attributed to the fact that C=O of esters generally absorb at higher frequency than the C=O of the corresponding acid. The COO^- anion has two bands at 1550 and 1420 cm^{-1}. Ketones and aldehydes also absorb at 1720 cm^{-1}. Quinone C=O absorbs at 1650 cm^{-1}, C=C of aromatic ring at 1600 cm^{-1} and 1510 cm^{-1} (ring breathing bands) and aryl esters absorb at 1720 cm^{-1}.

The total FA sample show a strong band at 1750 cm^{-1} which can be attributed to the C=O of COOH, ketones and aldehydes. Absorption bands at 1550 and 1420 cm^{-1} corresponding to the COO^- are not likely to be strong in the total sample since the spectrum was recorded on the solid FA which is known to contain very little ash or mineral constituents. A distinct shoulder is apparent between 1620-1640 cm^{-1} and this has usually been attributed to C=C vibrations of aromatic structures. There are some doubts regarding this assignment. First the 1510 cm^{-1} band characteristic of ring breaking

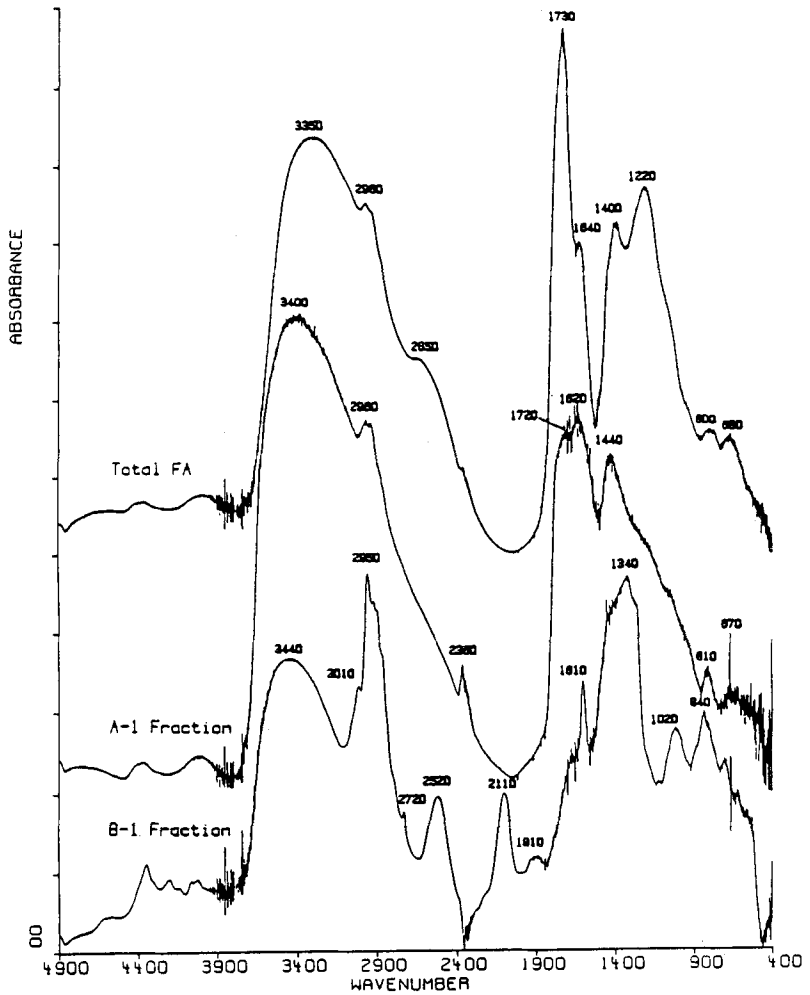

Figure 6 Solid state FT-IR spectra of solid total FA, hydrophilic fraction A-1 and hydrophobic fraction B-1.

is lacking. Second the band at 3030 cm^{-1} characteristic of CH stretch is not detected in the total sample.

The hydrophilic fraction show a weaker and broader band at 1720 cm^{-1}. Meanwhile the COO^- bands at 1560 and 1400 cm^{-1} are present. This may be attributed to the COO^- formed by interaction between the hydrophilic fraction and trace metals released during the HPLC separation.

The hydrophobic fraction shows a slight shoulder at 1720 cm^{-1} well defined sharp band at 1600 cm^{-1} and a slight shoulder at 1510 cm^{-1}. The 1600 cm^{-1} band can be assigned to α, β or α', β'- unsaturated ketones which are known to absorb in this region. There could be a minor contribution from aromatic C=C vibrations since the 1510 and 3010

cm^{-1} bands are only minor. The ^{13}C NMR, ^{1}H CRAMPS and UV -Vis scans of components of this fraction are in full agreement with this conclusion. Contribution of H-bonded conjugated C=O groups, to the band near 1610 cm^{-1} has been stressed by Theng and Posner [20].

Carbohydrate bands at 1050 cm^{-1} appear as a slight shoulder in the total sample and hydrophilic fraction. By contrast the hydrophobic fraction shows a well defined band at 1100 cm^{-1} which can be assigned to aliphatic alcohols, polysaccharides or to Si-O impurities.

ESR of Total FA and HPLC Fractions A-1 and B-1

ESR of the total sample showed a single symmetrical absorption line at g ~ 2.00 and spin content of 7.9 x 10^{17} [S] /g. The ESR spectra of the HPLC fractions A-1 and B-1 are shown in Fig. 7. The ESR spectrum of fraction A-1 shows several absorption lines with coupling constants of 71.4, 71.4, 59.6, 67.5, 65.6 and 71.4 gauss. This magnitude of coupling constants corresponds to field splitting by metallic protons. Line intensities do not follow a regular pattern which makes it difficult to identify exactly. The major absorption line correspond to the organic free radical and has a g value of ~ 2.0. These results suggest that the A-1 sample contains metallic paramagnetic ions possibly due to contamination from the HPLC system. Such contaminations are frequently reported in the literature. The inability to obtain a satisfactory ^{13}C CP/MAS spectrum for the A-1 sample, even though its weight is larger than that of the B-1 sample, is a further proof of the existence of paramagnetic contamination.

The ESR spectrum of the B-1 fraction shows a well defined absorption line of g ~ 2.00, due to organic free radicals. Additional weak absorption lines with coupling constants of 15 to 40 gauss are noted. It is not known whether these lines are caused by structures initially present in the B-1 sample or due to HPLC system contamination. It is

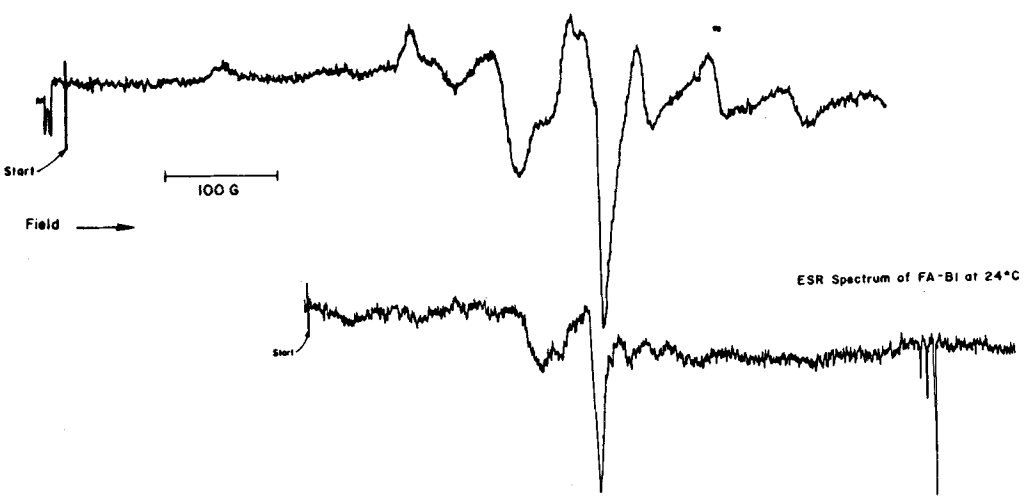

Figure 7 Solid state ESR spectra of hydrophilic fraction A-1 and hydrophobic fraction B-1 at 24 ±1°C.

remembered, however, that this fraction was collected using 85% CH_3OH as a mobile phase and the extent of metal contamination is expected to be less than in the A-1 fraction. The presence of organic free radical in this sample is also evident. Regardless of the paramagnetic contamination problem, the ESR spectra of both HPLC fractions indicated the presence of free radical structures. These results imply the presence of more than one type of organic free radicals in FA.

Summary and Conclusions

The yield from the preparative HPLC experiments of SR-FA was almost 70 percent. The hydrophilic constituents of FA represent ~ 40% of the total sample and can be resolved into six constituents each exhibiting featureless UV-Vis scans characteristic of aliphatic carboxylic acids and a few aromatic acids. The ^1H CRAMPS experiments confirmed the presence of acidic protons, strong hydrogen bonding and rigid structures. The presence of paramagnetic metallic contamination was indicated by the ESR and FT-IR spectra, and by the inability to obtain successful solid state ^{13}C CP/MAS spectrum. However, the ESR spectra indicated the presence of an organic free radical in this fraction.

The hydrophobic constituents of the FA represent ~ 30% of the total sample and can be resolved into 12 constituents as illustrated by the analytical chromatograms of this fraction. The UV-Vis scans of the peaks indicated the presence of repeated structurally related units. This is one of the first resolution of individual constituents of FA without chemical or thermal degradation. The UV-Vis scans of the repeated units were characteristic of conjugated aliphatic ketones and phenolic compounds. The ^1H CRAMPS and ^{13}C CP/MAS NMR spectra confirmed the predominant aliphatic structures in this fraction. The resolution in the ^1H CRAMPS spectra were comparable to those of model compounds. The reproductibility of proton distribution was excellent and indicated about 6% aromatic protons and 50% labile protons. Combined with evidence from the UV-Vis scans and the FT-IR, conjugated aliphatic ketones are likely to be significant constituents of this fraction. Contribution of H-bonded conjugated C=O groups in the FT-IR spectrum of this fraction provided further evidences to this conclusion. Free radical structures were also detectable in this fraction. Conjugated ketones and/or quinone structures are likely responsible for the presence of organic free radicals in this fraction.

The overall structure of SR-FA can be visualized as a mixed polymeric structure consisting of hydrophilic and hydrophobic subunits linked by weak interactions. The detection of several structurally related or repeated units in both FA fractions strongly suggests that FA is polymeric. Vanillic acid structures seemed to be present only in the FA macrostructure.

The forementioned picture of FA has some features that are in common with the published proposed models and formation pathways. However, it is not in full agreement with any single model. It is important to visualize the structure of FA only as a representation of a dynamic equilibrium at a given time. While the major structural units exhibit the same general properties, the contribution of each group to the macromolecule may vary. This approach would allow us to explain areas of homogeneity and heterogeneity in FA macromolecular structure.

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Fluorescence Spectroscopy as a Means of Distinguishing Fulvic and Humic Acids from Dissolved and Sedimentary Aquatic Sources and Terrestrial Sources

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Abstract

Thirteen fulvic acids (FA) and humic acids (HA) isolated from river waters and sediment, marine sediments, leonardite, soils, and paleosol, have been investigated by fluorescence spectroscopy in the emission, excitation and, partly, synchronous scan excitation modes. Emission spectra are generally characterized by a unique broad band, whereas excitation spectra exhibit a variable number of peaks or shoulders of various intensity; these peaks are particularly well-resolved for sedimentary HA samples. A decrease in the relative intensity of fluorescence, which is associated with a red-shift (longer wavelengths) of both the emission maximum and the main excitation peaks, is observed when passing from dissolved aquatic and soil FA to river and marine sedimentary HA, to leonardite and soil HA, and, finally, to paleosol HA. Evident differences are shown in the relative intensity and wavelength maxima, measured in any mode, between soil FA and HA from the same source. For FA and HA of various nature and origin, the fluorescence is suggested to be caused by chemically different structural units. These units fluoresce from the blue-violet to the green and consist of variously extended, condensed, aromatic and/or heterocyclic ring systems, with a high degree of electronic conjugation and bearing suitable hydroxyl, alkoxyl and carbonyl groups (e.g salicyl, cinnamic and hydroxybenzoic derivatives, naphtols, naphtoquinones, coumarin), and quinoline-derivatives, flavonoids and Schiff-base derivatives. Fluorescence properties of humic substances may represent an additional diagnostic criterium useful in distinguishing between FA and HA from the same or various natural sources.

Introduction

In the last two decades fluorescence spectroscopy has been applied extensively as a useful means of general chemical characterization and, in particular, identification

of certain structural and functional constituents (fluorophores) in natural, artificial and synthetic humic substances of various origin. With this method, the effects of concentration in solution and pH can also be taken into account. A comprehensive review on fluorescence of HS has been recently published [1]. However, only a few comparative studies are available, which discuss the potential of this technique to furnish spectra that can enable fulvic acid (FA) and humic acid (HA) fractions from the same source to be unambiguosly distinguished, and that can allow differentiation of FA or HA of various origin and genesis. In one of these studies, a marine sedimentary HA was found to fluoresce more intensively and showed emission and excitation fluorescence maxima at wavelengths longer than FA from the same source [2]. In another investigation, fluorescence excitation spectral shapes and maxima of FA and HA couples extracted from a large number of soils of widely differing geographic and pedologic environments, revealed apparent differences typically related to the soil nature and properties of the soil [3]. Sedimentary marine HA has been found to be characterized by excitation wavelength maxima lower than terrestrial HA, and both fluoresce less intensively than dissolved organic matter [4]. Emission fluorescence spectra corrected for Raman and Tyndall scattering have been presented for FA and HA extracted from estuarine particles. These spectra showed wavelength maxima higher than those measured for their counterparts in autochthonous marine sediments [5]. Recently, evidence has also been obtained of typical differences in fluorescence intensity and spectral shape and maxima between FA and HA and among humic substances of various sources, including soil, peat, leonardite and river water [6]. Even more recently, results of two comparative fluorescence studies have shown that terrestrial HA had the most pronounced emission and excitation maxima at the longest wavelength (green), followed by HA from lake and deep marine sediments, river and marine aquatic HA, river and marine surface sedimentary HA, and, finally, by terrestrial and aquatic FA, which showed fluorescence maxima at the shortest wavelength (blue-violet). When comparing terrestrial FA and HA, it has also been found that fluorescence spectra are characteristically well differentiated in shape, with maxima at wavelengths distinctly higher for HA than for correspondent FA. When comparing aquatic HA and FA, however, it was observed that spectra were less differentiated, with maxima centered at much closer wavelengths [7,8].

The principal objective of the present study was to furnish further evidence of the possibility of applying fluorescence spectroscopy to differentiate humic materials according to their origin and to distinguish between FA and HA from the same source. For this purpose, the fluorescence spectra of a number of FA and HA samples, isolated from various aquatic, sedimentary and terrestrial sources and previously characterized extensively for chemical and spectroscopic properties, have been comparatively analyzed and discussed.

Materials and Methods

The identification and origin of the thirteen fulvic and humic acid samples investigated in this study are furnished in Table 1. The samples were supplied by

various colleagues: the river aquatic FA (R29 to R32) by Dr. R.L. Malcolm (U.S.G.S., Denver, CO, USA); the leonardite HA (M20) and the Chino soil HA (M23) by Dr. J.P. Martin (Univ. of California, Riverside, CA, USA); the gley soil HA (NG5) by Dr. K. Yonebayashi (Prefectural Univ., Shimogamo, Kyoto, Japan); and the paleosol HA (P5) by Dr. G. Calderoni (Univ. La Sapienza, Roma, Italy).

Table 1 Identification and origin of fulvic acids (FA) and humic acids (HA).

Sample Identity	Origin	Sampling Place	Sampling Depth	Additional Information
R29, FA	Missouri River, water	Sioux City, Iowa,U.S.A.	surface	3.2[a]
R30, FA	Ohio River, water	Cincinnati, Ohio,U.S.A.	surface	3.2[a]
R31, FA	Ogeechee Stream, water	Grange, Georgia,U.S.A.	surface	8.1[a]
R32, FA	Yampa Stream water	Yampa, Colo.,U.S.A.	surface	1.0[a]
MS7, HA	Filiouris River, sediment	Delta area at Xilagani,Greece	0-40 cm	mud[b]
MS3, HA	Strymonikos Plateau, marine sediment	North Aegean Sea,Greece	0-60 cm	medium sand, gravel[b]
MS1, HA	Samothraki Plateau, marine sediment	North Aegean Sea,Greece	0-170 cm	silt, silty mud[b]
M20, HA	Leonardite deposit	Wyoming,U.S.A.	-	-
NG5, HA	Gley soil	Nagaoka, Niigata,Japan	0-15 cm	paddy[b]
M23, HA	Chino soil	Chino, Cal.,U.S.A.	0-15 cm	clay loam[b]
P5, HA[b]	Volcanic paleosol, exposed cliff	Procida Island, Thyrr.Sea,Italy	30 m	c
F4, FA	Brown Mediterran. soil	Sassari, Sardinia,Italy	0-15 cm	loamy sand[b]
H4, HA	Brown Mediterran. soil	Sassari, Sardinia,Italy	0-15 cm	loamy sand[b]

[a]DOC: Dissolved organic carbon (mg C/l) [9]. [b]Texture.
[c]Interbedded within pyroclastic rocks; age, 28,850±860 yr BP [13].

Methods of extraction and analytical properties of the FA and HA samples used have been previously described [8-14]. Some analytical data are presented in Table 2.

Sample solutions were prepared by dissolving each FA or HA in deionized water at a concentration of 100 mg/l. After overnight equilibration at room temperature (RT), solutions were filtered through Whatman No. 2 paper and adjusted to pH 8.0 with 0.05 N NaOH.

Table 2 Major elemental atomic ratios and some analytical data for fulvic acids (FA) and humic acids (HA).

Sample	C/H	C/N	O/C[a]	Ash	E_4/E_6	Free radical	Ref.
	Atomic ratios[b]			%	ratio	concent[c]	
R29, FA	0.91	54.82	0.56	<1.00	23.7	1.22	[10]
R30, FA	0.92	43.94	0.59	<1.00	15.9	4.79	[10]
R31, FA	0.98	47.50	0.61	<1.00	17.1	3.42	[10]
R32, FA	1.00	53.86	0.62	<1.00	18.8	1.06	[10]
MS7, HA	0.90	18.30	0.61	12.30	4.5	7.26	[8]
MS3, HA	0.65	10.10	0.46	18.20	5.3	1.18	[8]
MS1, HA	0.94	16.60	0.38	11.40	5.1	3.53	[8]
M20, HA	1.32	20.25	0.41	3.10	5.4	2.23	d
NG5, HA	0.85	12.79	0.48	<1.00[d]	5.0[d]	1.50[d]	[12]
M23, HA	1.01	13.89	0.40	2.30	5.2[d]	2.21[d]	[11]
P5, HA	2.38	33.33	0.42	2.20	5.0	7.03	[13]
F4, FA	0.49	16.24	0.95	14.20	6.2[d]	1.84	[14]
H4, HA	1.01	17.77	0.49	1.40	4.6[d]	16.55	[14]

[a]Oxygen calculated by difference. [b]Calculated on an ash- and moisture-free basis.
[c]Spins/g x 10^{-17}. [d]Present study.

Fluorescence spectra were recorded at RT on a Perkin Elmer LS-5 luminescence spectrophotometer equipped with a Perkin Elmer Data Station 3600. Data were generated and processed by Perkin Elmer PECLS programs. The emission and excitation slits were set at 5-nm band width and a 120-nm/min scan speed for both monochromators selected. The fixed scale setting was chosen according to the fluorescence intensity of each sample, so that the highest peak in the spectrum could approximate 90% relative intensity. Comparable values of relative intensity were then calculated for the various samples. Emission spectra were recorded over the range 380 to 550 nm at a constant excitation wavelength of 360 nm. Excitation spectra

were obtained over a scan range of 270 to 500 nm, by measuring the emission radiation at a fixed wavelength of 520 nm. Synchronous-scan excitation spectra were measured only for brown soil FA and HA, by scanning simultaneously both the excitation and emission wavelengths. The excitation wavelength was varied from 290 to 550 nm during the scans, while maintaining a constant wavelength difference of $\Delta\lambda = \lambda_{em} - \lambda_{exc} = 18$ nm, which was found to produce optimally resolved spectra.

Results and Discussion

Wavelengths of fluorescence maxima (peaks and shoulders) obtained in the emission and excitation modes and relative fluorescence intensities for FA and HA examined are listed in Table 3. Fig. 1 shows the fluorescence emission and excitation spectra of most examined FA and HA samples. For simplicity, the spectra of only one (the Ohio River FA) of the four river aquatic FAs is shown, because all four stream fulvic acids are very similar. Fluorescence emission, excitation and synchronous-scan spectra of FA and HA isolated from the brown soil are presented, in Figs 2 and 3, respectively, to allow an immediate visual comparison.

Fluorescence emission spectra measured at an excitation wavelength of 360 nm (Figs 1 and 2, Table 3) generally feature a broad band of relative intensity which decreases and a wavelength maximum which increases in the order: stream and soil FA, stream and marine sediment HA, soil and leonardite HA, paleosol HA. Sedimentary HA are characterized by flat fluorescence maxima with tails toward higher or lower wavelengths, whereas leonardite and soil HA show shoulders at lower wavelengths. Therefore, these results, together with comparable data reported in the current literature [1,4,6-8,15], permit the establishment of two first-evident parameters of distinction between FA and/or HA of various natural sources, based on the values of relative intensity and wavelength of the fluorescence emission maximum and supposing that the same excitation wavelength is maintained in all experiments.

Fluorescence excitation spectra (Figs 1 and 2) are apparently better structured than the corresponding emission spectra and confirm the trends previously described. FA samples show, in any case, a typical excitation peak at 390 nm (Figs 1a and 2a, Table 3). Comparable wavelengths have been measured by some authors [6,7], whereas others have obtained few lower values for FA of various origin [1,2,16,17]. In the case of soil FA, the peak at 390 nm is accompanied by an equally intense fluorescence at lower wavelengths and by additional secondary peaks and shoulders at higher wavelengths (Fig. 2, Table 3). This behaviour, however, does not seem to be of general validity for other soil and peat FAs, which often show, in addition to the previous peak, one or more intense peak(s) at higher wavelengths [3,6,7,16]. The excitation spectral shape of stream FA (Fig. 1a) evidently differs from that of soil FA in that it always shows a unique peak at around 390 nm. This peak is associated with shoulders on the low wavelength side [6,7].

In general the excitation spectra of sediment HA usually have three (or four) peaks of similar intensity in the low, intermediate and high wavelength ranges,

although the HA from deep marine sediment features more intense peaks at high wavelengths (Figs 1b-d; Table 3). Leonardite and soil HA generally show two close excitation peaks at high wavelengths, with a shoulder between 390 and 400 nm (Figs 1e, 1g, 2; Table 3). Gley soil HA, however, features an additional intense peak at 393 nm and weak shoulders in the low wavelength range (Fig. 1f). Paleosol HA is characterized by a unique excitation maximum centered at the highest wavelength (471 nm) among examined samples, with shoulders (Fig. 1h). These results, which generally agree with data reported in the literature for FA and HA of similar origin [2-4,6-8,15-17], make it possible to further distinguish between FA and/or HA on the basis of the shape and wavelength maxima of excitation fluorescence spectra.

Table 3 Fluorescence wavelengths (nm) of emission and excitation maxima and relative intensity (R.I., arbitrary units) measured for fulvic acids (FA) and humic acids (HA).

Sample	Emission peak		shoulders/	Excitation peaks		
	λem	R.I.	tail (--->)	Main[a]	Secondary[a]	Shoulders[a]
R29, FA	453	57	-	390	-	342,303
R30, FA	456	69	-	390	-	360,340
R31, FA	458	57	-	389	-	356,344
R32, FA	459	47	-	389	-	357,342
MS7, HA	462	14	--->515	306,442,391	-	462
MS3, HA	472	8	--->515	312,444,390	-	460
MSI, HA	505	10	--->460	448,463	391,330	310
M20, HA	510	2	490,470	465,451	-	396
NG5, HA	507	5	--->470	446,464	393	438,360,338
M23, HA	514	2	470	464	451	393
P5, HA	522	1	-	471	-	453,394
F4, FA	451	14	-	388,336,301	438,457	493
H4, HA	514	5	-	464,450	-	392

[a]Listed in decreasing order of relative intensity in the spectrum of each sample.

Comparison of FA and HA from the brown soil in regard to fluorescence emission and excitation spectral shape, relative intensity and wavelength (nm) maxima, (Fig. 2; Table 3), permits the two humic fractions to be easily distinguished. This difference in emission and excitation wavelength maxima is also apparent for other FA and HA couples of terrestrial origin, but it is not so evident when

comparing river or marine aquatic FA versus HA [5,7]. This is not surprising if one considers that the molecular, chemical and genetical characteristics of FA and HA from the same aquatic source are more similar than the same characters of their terrestrial counterparts. Synchronous-scan excitation spectra of soil FA versus HA (Fig. 3) feature even more differentiated spectra than those previously examined. This type of fluorescence spectra also appears to be promising for distinguishing between FA and HA of aquatic origin [7].

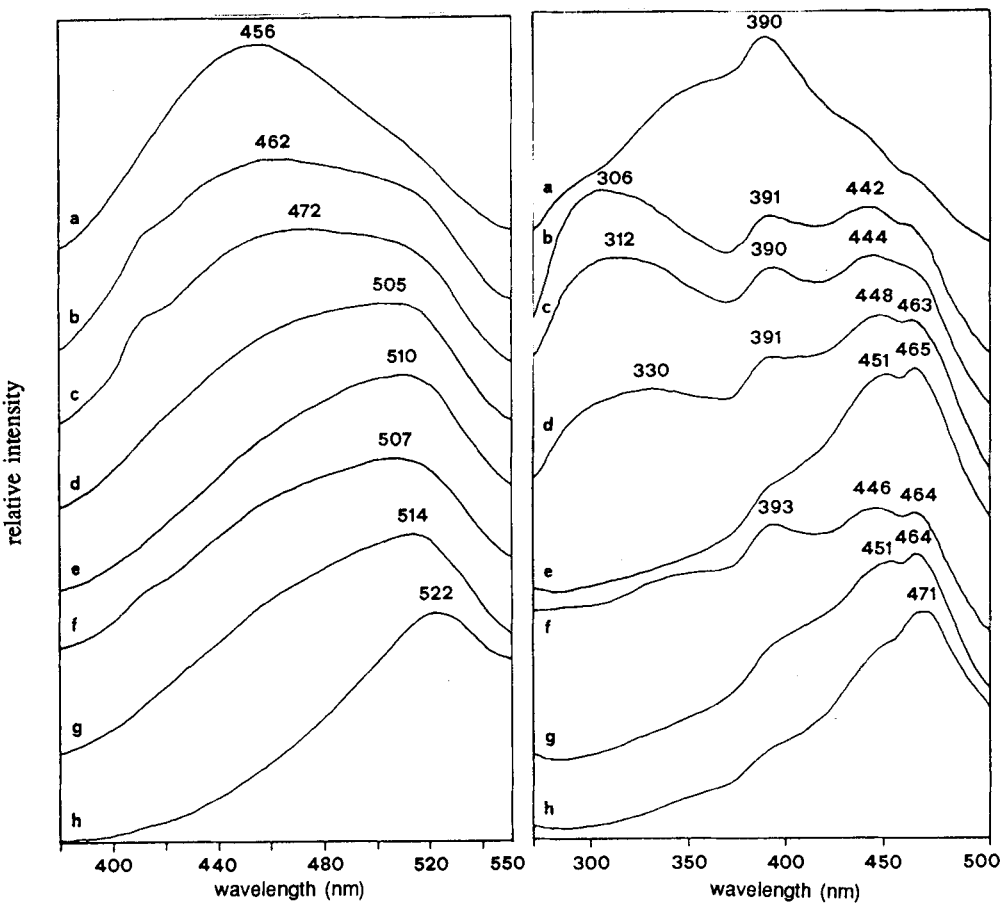


Figure 1 Fluorescence emission (left) and excitation (right) spectra. Ohio River FA (a); sediment HA from the Filiouris River (b), the Strymonikos Plateau (c) and the Samothraki Plateau (d); leonardite HA (e); gley soil HA (f), Chino soil HA (g), and paleosol HA (h).

Fluorescence, as is well-known, depends on the availability of delocalized electrons. For example, fluorescence occurs in the presence of condensed aromatic ring systems bearing, in suitable positions, electron-donating or accepting substituent groups, such as hydroxyls, alkoxides and carbonyls of various nature. It can also occur in the presence of conjugated unsaturated systems capable of a high degree of

resonance, i.e. of electron delocalization [18,19]. Because of the well-known chemical and structural complexity and heterogeneity of humic materials, only hypothetical identification of the relevant molecular constituents responsible for fluorescence in FA and HA may be made by comparison with fluorescence properties of chemically-defined molecules. This should possibly be carried out with the aid of other available analytical, spectroscopic and genetical information. In Table 4 a number of pure compounds and their fluorescence maxima are listed. These compunds may be expected to be potential contributors to the fluorescence of natural FA and HA, on the basis of similar structural units identified as products of hydrolysis and/or degradation of humic materials or units hypothesized as components of molecular structures proposed for them [20,21].

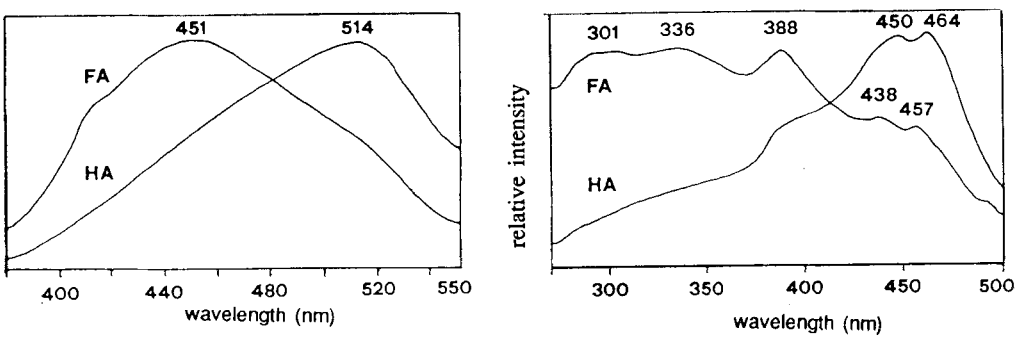


Figure 2 Fluorescence emission (left) and excitation (right) spectra of brown soil FA versus HA.

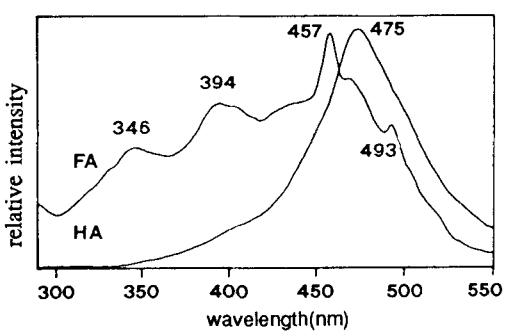
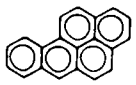
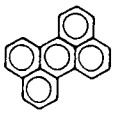
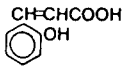
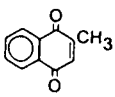
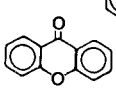
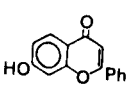
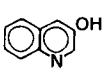


Figure 3 Synchronous-scan excitation spectra of brown soil FA versus HA.

As the number of aromatic rings condensed in a straight chain and the number of conjugated double bonds increase, the fluorescence emission wavelength increases [19,22]. Functional groups such as hydroxyls, alkoxides, carboxyls, aldehydes, ketones and esters, are also known to produce a red-shift in the emission wavelength [19,22]. This would explain the long fluorescence wavelength which characterizes terrestrial HA. Feasible contributors to fluorescence of terrestrial HA (Table 4) include: structural units such as aromatic rings condensed in long chains, methylnaphto-

Table 4 Fluorescence emission and excitation maxima of potential contributors, as structural units, to the fluorescence of fulvic acids (FA) and humic acids (HA) from soil, stream and marine environments.

Compound Name	Structural formula	λem max (nm)	λexc max (nm)	Ref.
1,2-Benzopyrene		400-500		[22]
Perylene		440,470		[22]
Methyl salicylate		448	302,366	[24]
Salicylaldheyde		525	327	[24]
Protocatechuic acid		455	340-370	[18]
2-Hydroxycinnamic acid		500	360	[24]
Caffeic acid		450	365	[24]
2-Methyl-1,4-naphtoquinone		480	-	[18]
Coumarins:				
unsubstituted		454	376	[22]
hydroxy- and methoxy-		450-480	320-365	[24]
esculetin		475	390	[24]
(6,7-dihydroxy-) scopoletin		460	390	[24]
(7-hydroxy-6-methoxy-) other disubstituted		449-515	367-420	[22,24]
Chromone-derivatives		445-490	320-336	[24]
Xanthone		456	410	[24]
3-Hydroxyxanthone		465	343,365	[24]
Flavones and Flavonoids		465-525	339-365	[24]
Schiff-base derivatives -N=C-C=C-N-		470	360-390	[23]
Hydroxy-quinolines		450-510	350-360	[18]

quinones, hydroxyquinolines and, particularly, coumarin derivatives and flavonoids, all derived from partial degradation of plant constituents and incorporated in the humic polymer. A blue shift in the fluorescence wavelength is observed when the aromatic system is branched or the straight chain is shorter [22]. Lignin degradation products (e.g coumarin derivatives, like esculetin, flavones, xanthones, and naphtoquinones) appear to fluoresce at wavelength maxima comparable to those of sedimentary HA (Table 4). The fluorescent chromophore group derived from browning reactions that

are polycondensation of carbohydrates and amino acids to melanoidins (the Schiff base derivative N=C-C=C-N), has also been suggested to be an important contributor to fluorescence of marine FA and HA [23]. These considerations support the idea that sedimentary humic materials have a mixed terrestrial and autochthonous origin.

Fluorescence of both terrestrial and stream FA may be attributed to simpler molecular units of low degree of aromatic polycondensation and rich in oxygenated functional groups, such as methylsalicylate units, e.g dihydroxybenzoic acids like protocatechuic acid, chromones, xanthones, and hydroxyquinolines, which fluoresce at short wavelengths (Table 4). The possible high contribution to fluorescence by coumarin and its derivatives (Table 4) supports the suggestion that both types of FA originate from plant materials [17]. No correlations can be found between fluorescence data and compositional or other analytical properties presented in Table 2.

Conclusions

Fluorescence spectral data presented in this paper appear adequate for distinguishing between FA and HA (particularly terrestrial) of common origin, and for distinguishing HA of various natural sources. The emission spectra appear featureless, but exhibit relative intensity and wavelength maxima different enough to provide a clear distinction among the various types of humic materials. The excitation spectra are more distinctive than the emission spectra, whereas synchronous-scan excitation spectra are particularly promising as diagnostic criteria of distinction.

Fluorescence properties of humic materials, in terms of structural units responsible for fluorescence, may be tentatively interpreted. This can be done by comparison with analoguous data available for pure compounds which have been identified, or hypothesized, as structural constituents of the humic molecules.

However, further studies, associated to information from other chemical and spectroscopic techniques, such as NMR, IR, GC/MS and ESR, are necessary in order to confirm and extend the preliminary classification of humic substances on the basis of fluorescence properties, as proposed in this paper.Fluorescence analysis is expected to provide important implications in the evaluation of the genesis and environmental behaviour and functions of humic substances.

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Dielectric Spectroscopy of Aqueous Solutions of Fulvic Acids

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Abstract

The complex dielectric spectrum of aqueous solutions (10% w/w) of fulvic and polymaleic acids has been measured at various frequencies between 1 MHz and 40 GHz. Similar to pure water a dispersion/dielectric loss region emerges in the range above 1 GHz. The measured spectra have been analytically represented by the empirical Cole-Cole relaxation spectral function to yield values for the extrapolated high- and low-frequency permittivity, the principal dielectric relaxation time, the relaxation time distribution parameter and the specific electric d.c. conductivity. In correspondence with aqueous solutions of synthetic organic molecules, the low-frequency permittivity of the fulvic acid/water mixtures is reduced, and the principal dielectric relaxation time is enhanced with respect to the pure water value. As with solutions of polyacrylic acid no solute contributions to the real part of the dielectric spectrum are found.

Introduction

As an important component of natural waters, humic substances play a significant role in geochemical and ecological processes. These acids act as complexing agents and, by this means, mobilize metal ions and organic pollutants in an aquatic environment [1,2]. Though some information exists on the physico-chemical and structural characteristics of humic substances in solution [3], many physical properties of these systems are still unclear. An open problem is the tertiary structure of humic substances in water. Other than with aqueous solutions of synthetic polymers, micellar or membrane-like aggregates have been recently proposed for the humic acid/water mixtures [4].

Aiming at information on specific solute-solvent interactions which might be associated with the tertiary structure of humic substances in water, we performed a dielectric relaxation study on aqueous solutions of fulvic acids (FA) and polymaleic acid (PA). The latter, a recently characterized [5-7] polycarboxylic acid formed by hydrolysis of base-catalyzed homopolymerized maleic anhydride, is assumed to be an interesting model of FA [8,9]. The microwave dielectric spectrum of aqueous solutions clearly reflects hydration properties of the solutes [10-18]. The evaluation of the measured spectra in terms of molecular models, however, is not unambiguously possible. We therefore restrict ourselves to a comparative discussion of the present data and of results for aqueous solutions of synthetic polymers, as well as of low-molecular solutes.

Materials and Methods

Samples

Two fulvic acids were isolated from the Bh horizons of Humic Haplorthod (podzol) located in Vollbüttel, West Germany (FA-PV) and Armadale, Canada (FA-PC), respectively. A third fulvic acid was isolated from water of a lake in Huelva, Spain (FA-T). This lake is surrounded by peatland. Details of the extraction and purification procedures have been published elsewhere [19-21].

The polymaleic acid (PMA-I) was prepared according to Braun and Pomakis [22] and subsequently acidified with a strong cation exchange resin in the protonated form. A purified PMA sample (free of pyridine and other aromatics) was obtained by redissolution of freeze-dried PMA-I in acetone and coagulation by addition of chloroform in excess. The coagulate was removed by filtration and dried at room temperature afterwards (PMA-II). The results of an elementary composition and a functional group analysis of the samples is presented in Table 1. C and H were determined by dry combustion, N by the automated Dumas method, and O was estimated from the difference. The total acidity was determined by the barium hydroxide method and the carboxyl groups by calcium acetate exchange, as previously described by Schnitzer and Khan [23]. Phenolic hydroxyls were considered to be equal to the difference between the total acidity and the carboxyl groups.

Table 1 Elementary composition (% w/w) and concentration of oxygen-containing functional groups (meq/g) of the humic acid samples. All values are calculated on a dry, ash-free humic acid.

Sample	C	H	N	0 + S	Total Acidity	COOH	Phen. OH
FA- PV	46.2	3.4	0.8	49.6	11.2	8.8	2.4
FA- PC	50.9	3.3	0.7	45.1	11.5	9.1	2.4
FA- T	43.4	4.2	1.3	48.9	12.7	7.6	5.1
PMA	45.9	4.4	0.6	49.1	12.0	8.8	3.2

Table 2 Concentration data (c_s, weight of solute per volume of solution; s, weight per cent), density ρ, molarity c_w of solvent, volume fraction v of solute, and pH value of the humic acid solutions at 25°C.

Solute	c_s g/cm³ ± 0.0002	s % w/w ± 0.02	ρ g/cm³ ± 0.004	c_w mol/l ± 2	v ± 0.004	pH ± 0.05
FA- PV	0.0990	9.59	1.033	51.8	0.065	1.80
FA-PC	<0.015	<1.5	≈1.002	>54.8	≈0.008	-
FA-T	0.0954	9.16	1.041	52.5	0.052	2.25
PMA-I	0.1000	9.70	1.031	51.7	0.066	2.75
PMA-II	0.1000	9.77	1.023	51.2	0.074	-

Aqueous solutions with a concentration of about 10 % w/w have been prepared of FA-PV, FA-T, PMA-I, and PMA-II by adding bidistilled water to a preweighed amount of the respective fulvic acid. FA-PC was less soluble (< 1.5 % w/w). Concentration data, density, and pH value of the solutions, as well as the volume fraction of solute, are given in Table 2.

Complex Permittivity Measurements

The complex (relative) electric permittivity (i, imaginary unit)

$$\varepsilon(\nu) = \varepsilon'(\nu) - i\varepsilon''(\nu) \qquad (1)$$

of the sample liquids has been determined as a function of frequency ν (Fig. 1) by frequency domain measurements [24]. The liquids have been exposed to harmonically alternating, weak electric fields to observe the responding dielectric polarization. Three different methods, which had been successfully applied in various previous studies on aqueous solutions, have been used to cover the frequency range between 1 MHz and 40 GHz.

From 1 to 100 MHz we utilized a sensitive rf-admittance bridge (Boonton 33D/1) to perform at seven fixed frequencies input impedance measurements on a small specimen cell. This cell contains the sample in a short piece of a circular waveguide, which is excited far below its cut-off frequency [25]. Modal analysis of the transition between the coaxial line feeder and the waveguide-below-cut-off section has been performed [26]. It was found that the cell can be represented by a simple equivalent network [26]. For the liquids under consideration ($\varepsilon'>60$) this network simply consists of two capacitors, the capacitances of which have been determined by calibration measurements with the empty cell and with water as reference liquid.

Between 1 MHz and 1 GHz a vector voltmeter (Rohde & Schwarz ZPU) has been additionally used to measure the transmission coefficient of a cell in which the sample is also placed in a waveguide-below-cut-off section [26]. This cell essentially consists of a coaxial line, the inner conductor of which is interrupted for a certain

distance to form a small piece of circular cylindrical waveguide. This waveguide contains the liquid sample between two dielectric windows. This "transmission" cell, in analogy to the "reflection" cell, has been likewise treated by modal analysis [26]. A π-network representation follows thereby. The values of the capacitors of this lumped-element circuit have also been found by calibration measurements.

At frequencies above 1 GHz a travelling-wave method has been applied. The wave transmitted through a liquid-filled circular cylindrical waveguide was balanced against a reference wave by a doublebeam interferometer technique [27-29]. Five small-band microwave bridges, consisting of standard coaxial line components or waveguide devices, were used to cover the frequency range.

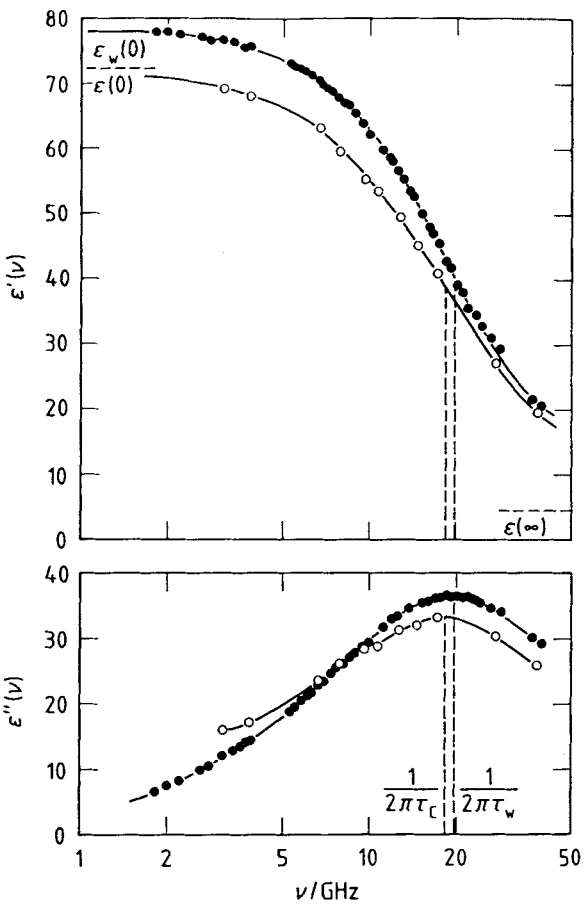

Figure 1 Real part $\varepsilon'(\nu)$ and negative imaginary part $\varepsilon''(\nu)$ of the complex (electric) permittivity plotted versus frequency ν for pure water (• [30]) and for the aqueous PMA-I solution (o) at 25°C. The curves represent the relaxation spectral functions eq. (3) and eq. (4), respectively, with the parameter values given in Table 3.

Experimental Errors

The experimental error in the complex permittivity data depends on the frequency of measurement and also on the applied method. Above 200 MHz it may be globally characterized by an uncertainty of ±2% for both ε' and ε''. Due to the high d.c. conductivity of the samples, measurements below 200 MHz were substantially affected by electrode polarization effects. We therefore restrict the following discussion to the microwave part (ν>200 MHz) of the dielectric spectra. Errors in the determination of the frequency ν were negligibly small. The temperature was controlled to within ±0.1 K during the measurements.

Results and Treatment of Data

In Fig. 1 the real part ε' and the negative imaginary part ε'' of the complex permittivity are displayed as a function of frequency ν for the PMA-I solution at 25°C. Also shown for comparison is the complex dielectric spectrum of pure water at the same temperature. The curves for the mixture resemble the corresponding curves for the solvent. Some differences, however, emerge, which are characteristic for all measured spectra of humic acid solutions. The extrapolated static permittivity $\varepsilon(0)$ of each solution is smaller than that of water, $\varepsilon_w(0)$. We shall comment on this effect below. The frequency $\nu_C = (2\pi\tau_c)^{-1}$ at which $\varepsilon''(\nu)$ adopts its relative maximum ($d\varepsilon''(\nu_C)/d\nu = 0$, $d^2\varepsilon''(\nu_C)/d\nu^2 < 0$) is different from that of water, $(2\pi\tau_w)^{-1}$. The dispersion region ($d\varepsilon'(\nu)/d\nu < 0$) of the mixture extends over a slightly broader frequency band. Finally, at low frequencies (ν<7 GHz) the $\varepsilon''(\nu)$ values of the solution exceed those of the pure solvent. This finding is an indication that the total loss is a sum

$$\varepsilon''(\nu) = \varepsilon_d''(\nu) + \sigma/(\varepsilon_0\omega) \qquad (2)$$

of a contribution $\varepsilon_d''(\nu)$ originating in dielectric relaxation processes and of another one resulting from the specific electric conductivity σ. In eq. (2), ε_0 denotes the electrical field constant and $\omega = 2\pi\nu$ the angular frequency.

Within the limits of experimental error, the microwave dielectric spectrum of pure water is characterized by a relaxation with one discrete relaxation time τ_w [30,31]. It can thus be represented by the Debye-type relaxation function [32] given by

$$\varepsilon(\nu) = \varepsilon_w(\infty) + [\varepsilon_w(0) - \varepsilon_w(\infty)] / (1 + i\omega\tau_w) \qquad (3)$$

The values of the parameters of eq. (3) at 25°C are given in Table 3.

Empirically, the dielectric spectra of the solutions can be described by the Cole-Cole relaxation spectral function [33]. The function

$$\varepsilon(\nu) = \varepsilon(\infty) + [\varepsilon(0) - \varepsilon(\infty)] / [1 + (i\omega\tau_C)^{(1-h)}] - i\sigma/\varepsilon_0\omega \qquad (4)$$

is therefore appropriate to analytically represent the frequency-dependent complex permittivity data. In this relation parameter h is a measure of the width of the underlying relaxation time distribution. The values of the parameters $\varepsilon(\infty)$, $\varepsilon(0)$, τ_C, h, and σ have been found by fitting eq. (4) to the measured spectra using a nonlinear least-squares regression analysis. The results obtained by this fitting procedure are also presented in Table 3. Also included in this table for comparison are data for a nearly l-molar aqueous solution of polyacrylic acid, PAA.

The PMA-II solution has been measured below 1 GHz only. In correspondence with the other samples no dispersion/dielectric loss region has been found in this frequency range. This finding is in accordance with previous results for aqueous solutions of PAA [34]. In contrast, solutions of salts of polyacrylic acid, like NaPA, at frequencies below about 1 GHz, clearly exhibit solute contributions in their dielectric spectra [34].

The solution of FA-PC has been measured at one frequency only (v = 6.0 GHz). For this reason parameters $\varepsilon(\infty)$ and h have been fixed at reasonable values in the above fitting procedure. By that means estimates for the extrapolated static permittivity $\varepsilon(0)$ and the principal dielectric relaxation time τ_C have been obtained.

Table 3 Parameters of the relaxation spectral functions for water (eq. 3) and for aqueous solutions (eq. 4) of fulvic acids, polymaleic acid, and polyacrylic acid at 25°C. Values marked by an asterisk (*) have been fixed during the fitting procedure.

Sample	s	$\varepsilon(\infty)$	$\varepsilon(0)$	τ	h	σ
	% w/w			ps		S/m
Water [29]	-	5.16±0.08	78.36±0.05	8.27±0.02	-	-
FA-PV	9.59	5.0±0.5	72.3±0.5	8.50±0.05	0.03±0.03	1.25±0
FA-PC	<1.5	4.7*	76.9±0.5	8.34±0.1	0*	0.5±0
FA-T	9.16	4.5±0.5	72.1±0.5	8.41±0.15	0.05±0.05	2.52±0
PMA-I	9.70	4.5±0.5	72.5±0.5	8.64±0.15	0.05±0.05	0.80±0
PMA-II	9.77	-	70.0±1	-	-	0.78±0
PAA [33]	6.95	5.5±0.5	73.0±0.5	8.69±0.15	0.02±0.02	0.17±0

Discussion

To look for potential special properties of the fulvic acid/water mixtures it is useful to compare the parameter values (Table 3) of the relaxation spectral function (eq. 4) with data for aqueous solutions of other organic solutes. For this purpose, the extrapolated static permittivity values of the fulvic acid solutions are plotted in Fig. 2 as a function of volume fraction v of the solute. Also presented in that diagram are data for aqueous solutions of non-dipolar small organic molecules, of PAA, and of other synthetic polymers. The latter solutes are dipolar themselves. The contributions of the solvents to the static permittivity are therefore used here as $\varepsilon(O)$ value. This

value should compare with the static permittivity of aqueous solutions of non-dipolar solutes.

As a result of the increasing dilution of the dipolar solvent by solute molecules there is a general tendency in the $\varepsilon(0)$ values to decrease with v. The static permittivity data of the fulvic acid and PAA solutions nicely fit into this trend (Fig. 2). There are no indications of special effects in the fulvic acid solutions. Such effects could be due to a particular shape of the solute particles or to uncommon hydration properties.

The principal dielectric relaxation time τ_C of the fulvic acid solutions is enhanced with respect to the pure water value τ_w. Such an enhancement, however, is a common feature of aqueous solutions of organic solutes [10-15, 17, 18, 35-38]. The shift τ_C-τ_w in the dielectric relaxation time of the present solutions has reasonable values. With the fulvic acid/water mixtures (10% w/w) it is even somewhat smaller than with the less concentrated PAA solution (7% w/w). We therefore conclude that the fulvic acids do not seem to induce unusual hydration water properties.

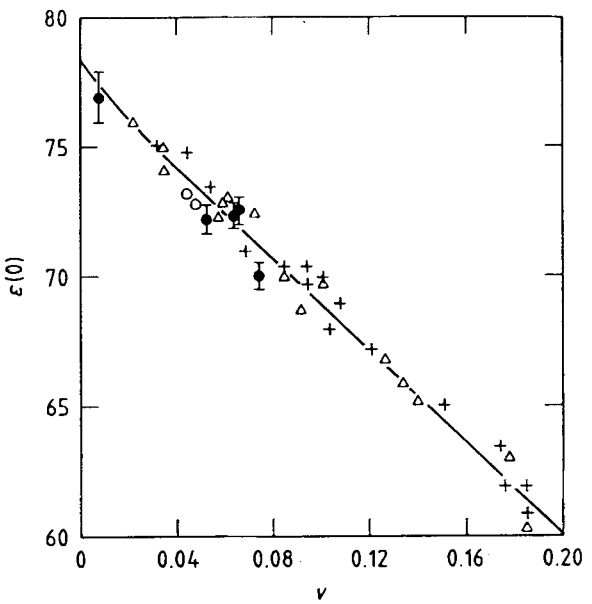

Figure 2 Plot of the extrapolated static permittivity $\varepsilon(0)$ as a function of the volume fraction v of solute for aqueous solutions of fulvic acids (•), polyacrylic acid (o) [34], nonionic synthetic polymers (Δ) [10,12,18] and small non-dipolar organic molecules (+) [35,39] at 25°C. With the solutions of dipolar synthetic polymers (Δ) only the solvent contribution to $\varepsilon(0)$ is considered here. The curve is hand drawn to indicate the trend in the data.

The specific electric d.c. conductivity σ of the fulvic acid solutions is higher than expected from the pH values. This enhanced conductivity is most probably due to the presence of small amounts of inorganic salts which, possibly, were not removed by the sample purification and preparation procedure.

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Cobaltihexamine as an Index Cation for Measuring the Cation Exchange Capacity of Humic Acids.

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Abstract

Cobaltihexamine (CoHM) is proposed as an index cation for measuring the cation exchange capacities (CEC) of "intact" and dissolved soil humic substances. Samples containing 2 to 5 mg humic substances were sufficient for each CoHM-CEC measurement. CoHM cation exchange capacity versus pH curves were better resolved than acid base titration curves in 0.1 M $NaClO_4$ and were slightly displaced towards lower pH values. Three adsorption maxima were obtained in the pH range 3 to 10, and these corresponded to functional group entities observed in $\delta pH/\delta ml$ curves from acid-base titration.

Introduction

One of the key problems in assessing the quantitative role of humic substances in transition metal ion binding is their quantitative measurement in terms of the diversity and the number of contributing species. Perdue [1] discussed the different methods for assessing the acidic functional group capacities of humic substances. Acid-base titrations are a classical means to measuring the potential number of complexing functional groups.

Methods based on the measurement of the metal ion complexation capacity may depend on the nature of the metal ion involved and generally lead to different complexation capacities [2]. The main disadvantage lies in the fact that these cations are hydrolysable, which leads to unreliable measurements in a broad pH range due to the fact that their adsorption mechanism is still a matter of discussion [3,4].

In previous contributions [5,6]) we reported on the use of silver(thiourea)$_n$ ($Ag(TU)_n$) for measuring the cation exchange capacity (CEC) of natural organic matter (NOM) in soil extracts. In the present study we examined the use of cobaltihexamine (CoHM) as an alternative means of measuring the CEC of the more hydrolyzable metal cations; this method takes advantage of the coagulating power of CoHM, which is similar to that of the $Ag(TU)_n$ complex.

The CoHM method was demonstrated for two different cases: a) for soil organic matter in its original form ("intact" or "in situ"), as it occurs in the Bh horizon of a Podzol soil, and b) for small concentrations (2 to 5 mg) of Podzol Bh soil extracts.

Experimental

Samples

Samples of the Bh horizon of a Podzol soil (Podzol Bh) were taken in Kalmthout (Belgium). Podzol Bh was essentially sand with an organic carbon content of 4.1±0.3% (10 determinations) [7] or 7% organic matter. The sample contained only traces of clay minerals. No amorphous or crystalline iron oxides were detected by either the NH_4-oxalate [8] or the Na-dithionate [9] method. The organic matter contained 58% humic acid, 12% fulvic acid and 30% humin [10]. The soil organic matter was considered as the dominant adsorption sink.

Extracts of Podzol Bh were obtained by repeated (3 times) two-hour mixing of 100 g Podzol with 1000 ml of 0.01 M NaOH, followed by 30 min centrifugation at 10000 rpm. The collected supernatants were dialysed using a Minitan ultrafiltration unit (Millipore), and the fraction $>10^5$ Dalton was used. The extract was salt-free and had a pH of 6 (Na^+-NOM22 and Na^+-NOM23). (The numbers refer to our laboratory classification). The E_4/E_6 ratio of the extract was 5, indicative of humic acids [11]. In another procedure the collected supernatants were used for the preparation of humic acid samples following the conventional humic acid precipitation in 0.01 M HNO_3. The humic acids were neutralized with NaOH prior to salt free dialysis (Na^+-HA21). Stock suspensions of about 1000 ppm of Na^+-NOM22 (82.5% NOM and Na^+-NOM23 (82.6% NOM) and Na^+-HA21 (83.4% NOM) were stored at 4°C in the dark.

Procedures

Cobaltihexamine (CoHM) spiked with ^{60}Co was used to monitor all CoHM adsorption equilibria. Since CoHM is an inert complex with an extremely low ligand exchange rate [12], it was labelled by adding 0.5 mCi ^{60}Co during its synthesis. The procedure of Bjerrum [13] was followed. A 0.01 M CoHM solution was stable in the pH range 2 to 12. Outside this range precipitation may occur.

All adsorption equilibria were run overnight at room temperature, followed by centrifugation (20 min. at 14000 rpm). Initial and supernatant solutions were radioassayed for ^{60}Co (Packard gamma scintillation counter), and the equilibrium pH was measured. The organic matter concentrated in the supernatant solutions was determined at 280 nm using an LKB Ultraspec K. The absorbances were corrected for the contribution of CoHM absorption to the optical density of the sample.

CoHM adsorption isotherms at constant pH were obtained by mixing 1 g of Podzol Bh with solutions containing known CoHM (spiked with ^{60}Co) concentrations

and which were brought to a constant electrolyte concentration (0.05 M NH_4Ac or 0.1 M of either KNO_3, NH_4Ac or $NaNO_3$). The pH was maintained at predetermined values by repeated small additions of 0.1 M KOH or NaOH in order to limit exposure to pH values exceeding the equilibrium value by more than 0.5 pH units.

CoHM-CEC versus pH curves were obtained from single batch experiments under N_2 atmosphere and in the absence of background electrolyte. 1 g of Podzol Bh was mixed with 30 ml solutions of varying CoHM concentration (which corresponded to a 5 to 10 fold excess with respect to the final CoHM-CEC) and was held overnight (16 hours) at the desired pH values by using an automatic titrating unit (Radiometer) and 0.1 M NaOH or 0.1 M HCl. The amount of 0.1 M NaOH or HCl used was recorded. A similar titration procedure on separate 1 g batches of Podzol Bh in absence of CoHM served as a reference acid-base titration.

A CoHM adsorption isotherm on the humic acid fraction (Na^+-HA22) of a Podzol Bh extract was obtained by mixing 5 ml portions of the extract (≈5 mg) with CoHM solutions (spiked with ^{60}Co) of varying concentration. The final concentration of the equilibrium solutions was 0.05M NH_4Ac, and the pH was 6.85.

A stock suspension (≈1000 mg/l) of the considered Podzol Bh extract was slowly titrated with acid or base to different pH values. Separate 5 ml portions (containing approx. 5 mg NOM) were withdrawn at regular pH intervals and were equilibrated with CoHM solutions (brought to the same pH) containing a 5 to 10 fold excess of CoHM, with respect to the estimated maximum adsorption capacity at the considered pH. The CoHM-CEC-pH function was obtained in absence of supporting electrolyte on Na^+-NOM23.

Acid-base titrations were performed under N_2 on 100 ml solutions containing a known amount (48 mg) of Na^+-HA21 ($MW > 10^5$) in 0.1 M $NaClO_4$. These solutions (acidified to a start pH of 2.94) were titrated from acid to base and vice versa using 0.1 M NaOH or 0.1 M $HClO_4$, respectively. The titrating solutions were added as 0.05 ml increments; the pH readings were done when the change was smaller than 0.01 units/min. The whole titration took about 8 hours.

Results and Discussion

Adsorption Isotherms

Examples of CoHM adsorption isotherms on Podzol Bh and Podzol Bh extract obtained in 0.05 M NH_4Ac are shown in Fig. 1. The adsorption isotherms were plotted as the amount adsorbed versus the amount added. This was done to demonstrate the general feature that CoHM adsorption reached a maximum after adding a 3 to 5 fold excess, with respect to the maximum adsorption capacity. The maximum was identified with the CoHM cation exchange capacity (CoHM-CEC) at the specified pH.

Adsorption of CoHM resulted in the gradual coagulation of the organic matter. The organic matter concentrations remaining in the supernatant solution, after phase separation, are shown in Fig. 1 for Podzol Bh and Na^+-NOM22. These concentrations gradually decreased with increasing CoHM additions. Precipitation was complete upon addition of 1 symmetry value of CoHM. In the case of Podzol Bh, the organic matter content of the equilibrium solutions (0.5 mg at the lowest CoHM concentration) was negligible compared to the total organic matter content (70 mg), which made corrections unnecessary. Coagulation of organic matter extracts, however, gradually increased from zero to complete precipitation. The amount of CoHM adsorbed was therefore corrected for organic matter precipitation (using optical density) and was expressed per gram of precipitated organic matter. The CoHM-CEC measured in 0.05 M NH_4Ac equalled 3.76 meq/g ash-free NOM or 3.1 meq/g NOM (pH=6.90), which agreed with the value of 3 meq/g found in $Ag(TU)_n$ measurements [6] on a similar extract.

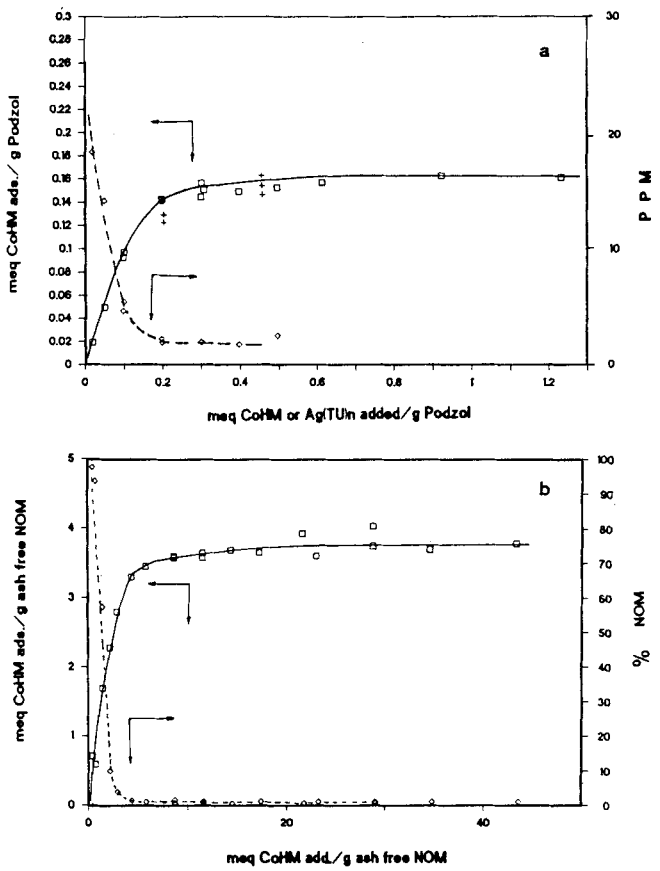

Figure 1 CoHM adsorption (meq/g) versus the amount of CoHM added (meq/g). a) Podzol Bh at pH 5.6 ± 0.1 (□). The concentration of organic matter (◇) in the equilibrium solution is also shown. Data for $Ag(TU)_n$ (pH 5.7±0.1) are included for comparison (+). b) Podzol Bh extract (Na^+-NOM 22) at pH 6.8±0.1. The % organic matter remaining in the equilibrium solution after phase separation is also shown. (Electrolyte concentration = 0.05 M NH_4Ac).

The CoHM-CEC for Podzol Bh equalled 0.162 meq/g at pH 5.6±0.1 and 0.05 M NH_4Ac. This was identical to the $Ag(TU)_n$ CEC determined under similar conditions of 0.05 M NH_4Ac and equilibrium pH (5.7±0.2) (see Fig. 1). Previously measured values for the $Ag(TU)_n$ CEC of a $CaCO_3$-amended Podzol (4 mg/g or ± 0.08 meq Ca/g) sample [6] in 0.1 M NH_4Ac buffer at pH=6, amounted to only 0.06 meq/g. This difference was easily explained by the much lower carbon content of the former sample (1.62%) as compared to the latter (4.06±0.2%).

CoHM isotherms were obtained on Podzol Bh at different pH values and 0.1 M electrolyte concentration (KNO_3 or NH_4Ac) (data not shown). The CoHM-CEC corresponding to the plateau of adsorption, increased with increasing pH as expected. The concentration of CoHM at equilibrium necessary to reach maximum adsorption (within 5%) ranged from $5x10^{-3}$ M at pH 3.5 to $1x10^{-3}$ M at pH 7.

It was sufficiently evident from the foregoing adsorption curves that CoHM-CEC values can be measured in both intact NOM of Podzol Bh and in NOM-extracts, when using a sufficient excess of CoHM (3 to 5 times the CEC). It should be stressed that CoHM-CEC measurements are possible on extracts containing about 2 to 5 mg organic matter, similar to observations made with both $Ag(TU)_n$ and CoHM on a commercial HA [6].

CoHM-CEC-pH Functions

CoHM-CEC values determined at different pH values using a sufficient CoHM excess are shown in Fig. 2 for Podzol Bh and a Podzol Bh extract, respectively. Fig. 2a shows CoHM adsorption data obtained under N_2 atmosphere in absence and presence of 0.1 M KNO_3 by overnight equilibration under careful pH control and using a 5 to 10 fold excess of CoHM. The adsorption maxima read from curves similar to Fig. 1 are also indicated. The CoHM-CEC values gradually increased from 0.060 meq/g at pH 2, to 0.225 meq/g at pH 7, and to 0.5 meq/g at pH 10. The CoHM-CEC-pH curves clearly showed different adsorption plateaus (see later).

The CoHM-CEC *vs* pH curve of the Podzol Bh extract in Fig. 2b shows adsorption plateaus similar to the observations made on Podzol Bh. CoHM-CEC values ranged from about 1.8 meq/g (at pH=3) to about 5.7 meq/g ash- free organic matter (at pH=9). Similar curves were obtained for other Podzol extracts.

Influence of Oxygen and pH History.

Fig. 2a demonstrates that under mild conditions of pH (pH 3 to 10), short term exposure of Podzol Bh to oxygen did not affect the CoHM-CEC, since the curves observed in a titration vessel under N_2 coincide with the data obtained in closed polycarbonate centrifuge tubes without special precautions to exclude air. All solutions were previously flushed with N_2. Such a result is in line with observations of Swift and Posner [14] and Borggaard [15], who found that no CEC variations occur, even under extreme conditions of pH, when working under N_2.

The pH history of the sample may, however, influence the available CEC by releasing blocked sites [16]. In an additional experiment it was indeed shown that the rapid addition of 10 ml 0.1 M NaOH to 1 g of Podzol Bh, equilibrated in a closed tube for 1 to 4 days, resulted in similar CoHM adsorption isotherms at pH 7. These isotherms, however, showed an enhanced CoHM-CEC equal to 0.390 meq/g.

The CoHM-CEC-pH functions of the soil extracts were not expected to change with the direction of the titration, since these extracts were obtained at high pH (12 to 13), and therefore no further solubilization and/or degradation and/or hydrolysis was expected in the considered pH range (3 to 10).

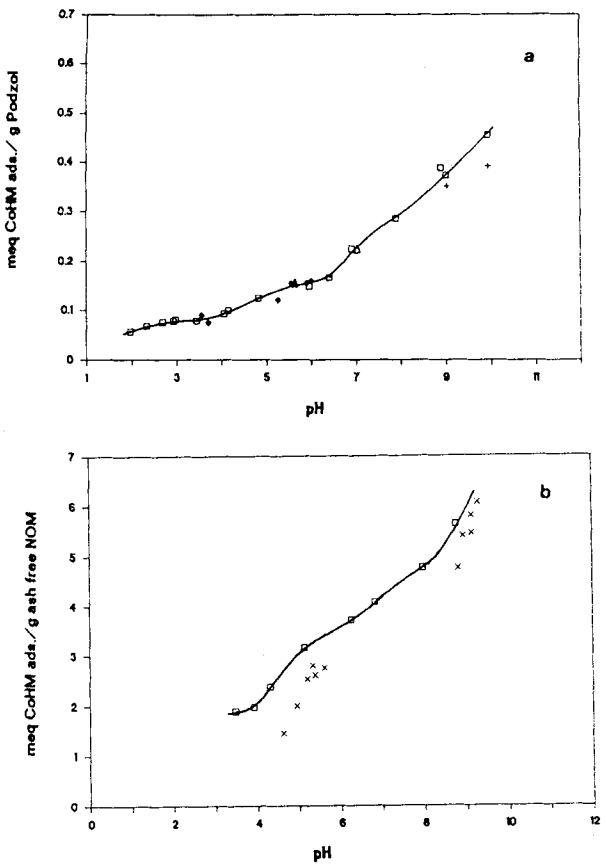

Figure 2 CoHM-CEC (meq/g) versus pH. a) Podzol Bh under N_2 atmosphere in absence (□) and presence of electrolyte: 0.1 M KNO_3 (+) and 0.05 NH_4Ac (Δ). CoHM-CEC values from maxima of adsorption isotherms are also indicated: 0.1 M KNO_3 (◆) and 0.05 M NH_4Ac (▲). b) Podzol Bh extract (Na^+-NOM 23) in absence (□) and presence (x) of increasing NaClO4 concentrations. From top to bottom: 0.0; 0.02; 0.05; 0.1; 0.25; 0.5 N (around pH 5) and 0.0; 0.02; 0.05; 0.1; 0.25 (around pH 9).

Adsorption Stoichiometry

Since CoHM was used as the chloride salt, the formation and adsorption of bivalent $Co(NH_3)_6Cl^{2+}$ complexes might influence the CoHM-CEC values. However,

CoHM-CEC values obtained in different $NaNO_3$/NaCl mixtures of 0.1 total molarity and pH 5.35 were identical, indicating that, essentially, $CoHM^{3+}$ is the adsorbing cation.

The mechanism of the CoHM adsorption process on Podzol Bh could be inferred from Fig. 3, which compares the CoHM-CEC function obtained under N_2 and in absence of background electrolyte, with the number of OH ions consumed to reach the different pH values in the presence and absence of COHM. The data refer to overnight equilibrium using a pH-stat in order to avoid variations due to the release of blocked sites. The starting pH of the system was 3.7. The Podzol Bh can be considered as being in the H^+-form. It was clear from Fig. 3 that the charge measured by acid-base titration in the presence of CoHM equalled or slightly exceeded the number of equivalents of CoHM adsorbed. The dissociated charge was therefore balanced by $CoHM^{3+}$ cations corresponding to a $3H^+$-$CoHM^{3+}$ stoichiometry in the considered pH range (2 to 10).

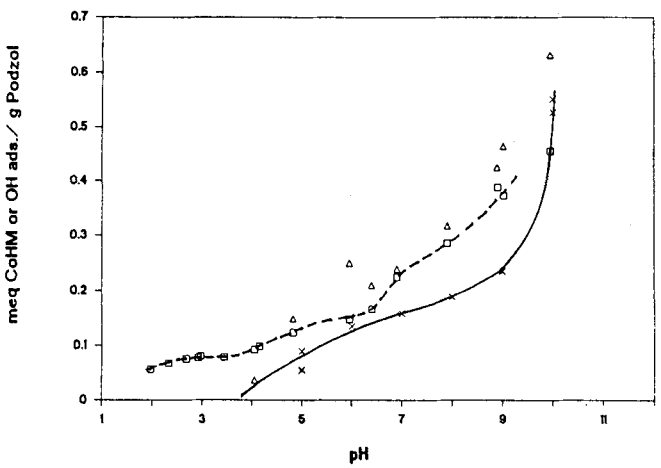

Figure 3 Comparison of CoHM-CEC-pH function (□) obtained by overnight equilibriation under N_2 with the number of OH-ions consumed to reach different pH values in absence (x) and in presence (Δ) of an excess of CoHM. Starting pH=3.7.

Influence of Ionic Strength and Counter Ion Effects

Inspection of Fig. 2a revealed that M^+-salt concentrations up to 0.1 M barely affected the CoHM-CEC values in the low pH range. Beyond pH 7 the CoHM-CEC decreased by about 8% upon increasing the salt concentration from zero to 0.1 M. This observation can be explained by the $CoHM^{3+}$-M^+ ion exchange behaviour of Podzol Bh. Indeed, using selectivity coefficients calculated for the different adsorption curves in 0.1 M M^+-salts at pH 3.55 (lnKc = 5) and pH 6 (lnKc = 9),

one predicts a 6% decrease in CoHM occupancy upon increasing the M^+-salt concentration from 0.001 M to 0.10 M. Such differences amounted to 0.005 to 0.010 meq/g and were of a magnitude similar to the experimental uncertainties observed in this pH region.

The presence of about 0.005 meq Ca/l, which was of a magnitude similar to the Ca concentations present in soil solutions, had no effect on the CoHM-CEC, since the maximum adsorption capacity, determined at pH 6.95 in presence and absence of 0.005 meq/l $Ca(NO_3)_2$, equalled 0.225 meq/g.

A similar CoHM-CEC dependency on M^+-salt concentration was observed for NOM-extracts as shown in Fig. 2b. A decrease of about 8% in CoHM-CEC was again observed upon comparison of measurements in zero and 0.1 M $NaClO_4$.

In conlusion, it may be stated that CoHM-CEC values depended on ionic strength. The process could essentially be understood as an ion exchange competition.

CoHM probably did not displace Fe present in NOM. Indeed, interaction constants at low to intermediate CoHM occupancies on NOM extracts were estimated to equal $10^{4.5}$ at pH 7. Interaction constants for Fe^{3+} with NOM were estimated to be 10^{12}-10^{14} [17]. CoHM was therefore unable to displace Fe^{3+} from soluble and intact NOM. Comparison with interaction constants for Ca ($10^{3.5}$ at pH 5), Mg ($10^{2.15}$ at pH 4.5) [18] and Na and K ($\approx 10^{2.6}$) [19], also leads to the conclusion that equilibrium concentrations in the order of 5×10^{-3} M CoHM were more than sufficient to displace the alkali- and alkaline earth metal ions associated with NOM present in soil extract and intact soils. The CoHM method can therefore be used to obtain a first estimate of the available CEC.

Comparison with Acid-Base Titration

Careful inspection of the CoHM-CEC-pH curves of Podzol Bh and Podzol Bh extracts in Figs 2 a and b showed three regions of adsorption in both cases. Maxima were observed in the pH ranges 3.5 to 4, pH 5.5 to 6.5 and beyond pH 8. It is suggested that these regions correspond to the titration of two carboxylic type groups and one OH-type group, by analogy with generally accepted assignments.

The CoHM-CEC-pH functions were similar to conventional acid-base titration functions and are compared with them hereafter.

Comparison of the CoHM-CEC-pH function with the discontinuous titration curve of Podzol Bh in absence of CoHM in Fig. 3 revealed that the CoHM adsorption curve was displaced towards lower pH values. CoHM adsorption therefore occurred with the displacement of a number of protons. The data in Fig. 3 strongly suggest that the functional group capacity obtained from acid-base titration around pH 7 identifies with the CoHM-CEC value obtained at the plateau at pH 6.

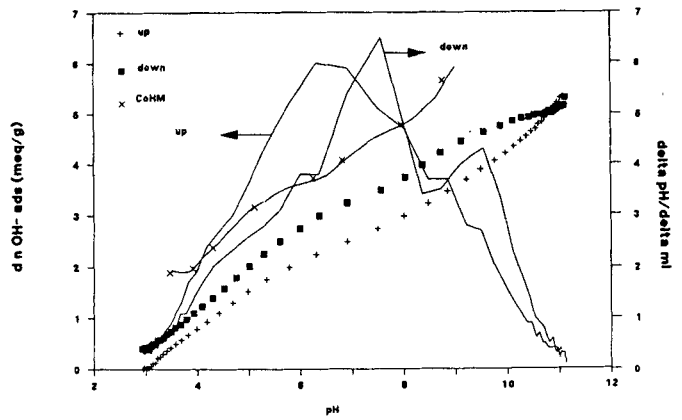

Figure 4 δn_{OH} (meq/g) and δpH/δml for the titration of Na^+-HA 21 in 0.1 M $NaClO_4$ background electrolyte. Starting pH=2.94. Forward (+) and backward (▪) titrations are shown. The CoHM-CEC (meq/g) versus pH curve (x) for Na^+-NOM 23 is shown for comparison.

Comparison of the CoHM-CEC-pH function with the continuous titration curves of Podzol Bh extract in Fig. 4 also revealed (by assuming zero charge at pH 3) that the CoHM adsorption curves were displaced towards lower pH values. The δpH/δml curves clearly show that three distinct functional groups with charges of 4.7 (pH 9.5), 3.5 (pH 7.5) and 1.6 meq/g (shoulder at pH 4.5), respectively were involved in the back titration. These data are comparable to the CoHM-CEC values of 5.0 meq/g (pH 8), 3.5 (pH 6) and 1.9 (pH 3.5).

The foregoing data strongly suggest that identical values of the various functional group capacities present in NOM were obtained from CoHM adsorption and acid-base titration but that equivalence was reached at lower pH values in the case of CoHM. Further research is necessary to confirm this observation.

The correspondance of the functional group capacity in Podzol Bh obtained from the Ca-acetate method (0.218 meq/g) and from the CoHM-CEC at pH 7 (0.225 meq/g) may be misleading. Indeed, comparison is in fact only allowed with CoHM-CEC values corresponding to the adsorption plateaus at pH 6 to 6.5 (CEC = 0.16 meq/g) or at pH 8 to 8.5 (CEC = 0.28 meq/g). Either choice fails to correspond to the Ca-acetate method.

Conclusions

CoHM adsorbs as a trivalent cation with dissolved and "intact" soil organic matter in the pH range 2 to 10. The adsorption plateau is reached at equilibrium concentrations of about 10^{-3} to $5x10^{-3}$ M CoHM and can be identified with the CoHM cation exchange capacity.

Dissolved soil humic acids are completely flocculated upon addition of 1 symmetry value of CoHM. This phenomenon allows easy phase separation and measurement of the adsorbed CoHM.

The CoHM-CEC-pH functions show the characteristic pattern of acid-base titration functions but are better resolved and are displaced towards lower pH values. At least three adsorption maxima are observed in NOM of intact Podzol Bh and in humic acids from a Podzol Bh extract. The nature of the functional groups involved cannot be identified and remains speculative, similar to the situation with acid-base titrations. The maximum of the CoHM-CEC-pH curve in the pH range 6 to 7 is suggested to be a good measure of the carboxylic functional group capacity. Functional group capacities obtained from CoHM-CEC-pH curves and acid-base titration data, appear to correspond, but should be critically evaluated in further research. Indeed, acid-base titration curves can depend on the NOM concentration and show hysteresis effects. Hydrogen bonding has been invoked [20] to explain these effects. CoHM-CEC-pH curves are independent on the NOM concentration. Increasing the ionic strength to 0.1 N monovalent salts leads to a 5 to 10% decrease in the CoHM-CEC, due to ion exchange competition. These changes are similar to observations made on acid-base titrations.

Although CoHM and $Ag(thiourea)_n$ adsorption lead to similar functional group capacities [6], CoHM is preferred over $Ag(thiourea)_n$. Indeed CoHM is a so-called inert highly stable complex, that can be considered as a non hydrolysable cation in the pH range of interest. In contrast, $Ag(thiourea)_n$ solutions are unstable beyond pH 8 and are subject to photochemical reactions. The advantage of using a non hydrolysable cation relates to the fact that methods based on the measurement of the complexing capacity using hydrolysable metal cations may depend a) on the nature of the cation and b) its ability to cause the release of a number of otherwise non titratable protons. CoHM on the contrary measures the charge imposed by the acid-base equilibrium alone.

The CoHM-CEC method is especially suited for determining small (2 to 5 mg), humic acid concentrations with a precision better than 5%. Samples containing less than 2 to 5 mg humic acid are less reliable, because the CoHM-CEC is determined as a difference of an initial and a final CoHM concentration, and because the necessary added CoHM excess needs to be sufficient for obtaining an equilibrium concentration of $\pm$ $5x10^{-3}$ M CoHM to reach the adsorption plateau.

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Investigation of Humic Acid Samples from Different Sources by Photon Correlation Spectroscopy

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Abstract

Photon correlation spectroscopy (dynamic light scattering) indicated that relatively large scatterers (50-200 nm diameter) are present in a number of soil, lake and ground-water humic and fulvic acids, as well as in natural waters of high humic content, but absent in 'synthetic' humic acid. The influence of ionic strength, Ca(II), La(III), EDTA, fluoride ions, surfactants, and ultrasound on size and zeta potential of these scatterers has also been investigated.

Introduction

In order to predict the transport behavior of humic acids toward metal ions in porous media (clay and rocks) it is important to know the actual size of humic and humic/metal aggregates. Retention on rocks and clay may depend on the surface (zeta) potential of these aggregates.

The size of humic and fulvic acid (H/FA) molecules and/or aggregates has been the object of extensive investigations. Earlier studies were based on osmometry, viscosimetry, and ultracentrifugation [1]; size-exclusion chromatography [2], ultrafiltration [3], flow field-flow fractionation [4], small angle X-ray scattering [5,6] and time-dependent fluorescence depolarization [7] have been employed more recently. Published results for average molecular weights (M_w) range between 200 and 100,000 daltons. Many authors stress the extreme heterodispersity of humic acids, and the presence of a significant distribution tail toward larger molecular sizes has been consistently reported in size-exclusion chromatography.

Little is known of the influence of metal ions or other factors on the size and surface characteristics of H/FA molecules/aggregates. Whereas the idea of a colloidal [4] or micellar [8] nature of humic acid solutions is not new, its colloidal properties have been the object of only limited studies.

Photon correlation spectroscopy (PCS, also known as dynamic light scattering, quasi-elastic light scattering or laser doppler velocimetry) is a relatively new, powerful tool for the non-destructive investigation of particle size and shape in the 1 nm - 10 μm diameter range [9]. When coupled to electrophoresis, PCS allows the determination of

surface (zeta) potentials [10]. A characteristic feature of PCS is that, since the signal intensity is proportional to the third power of particle size (z-weighting), it is more sensitive to the larger particles in a system.

Materials and Methods

A 3 W CW Argon ion laser was installed on a commercial PCS instrument (Malvern 4700) in order to achieve µg/l-level sensitivity (Fig. 1). The manifacturer's software was used to acquire the autocorrelation function of light scattered by the samples (128 channels, pseudo-logarithmically spaced). It is well known [11] that the autocorrelation function of coherent light scattered by a suspension of particles in Brownian motion can be described as a sum of exponential forms:

$$G(\tau) = A\,(1 + \beta\,\Sigma x_i\, e^{-2q^2 D_i \tau})$$

where A and β are, in practice, empirical constants, q is a function of wavelength and scattering angle, and x_i is the intensity of the signal due to particles of diffusion coefficient D_i. Particle hydrodynamic radius can be estimated from diffusion coefficients using the Stokes-Einstein relationship:

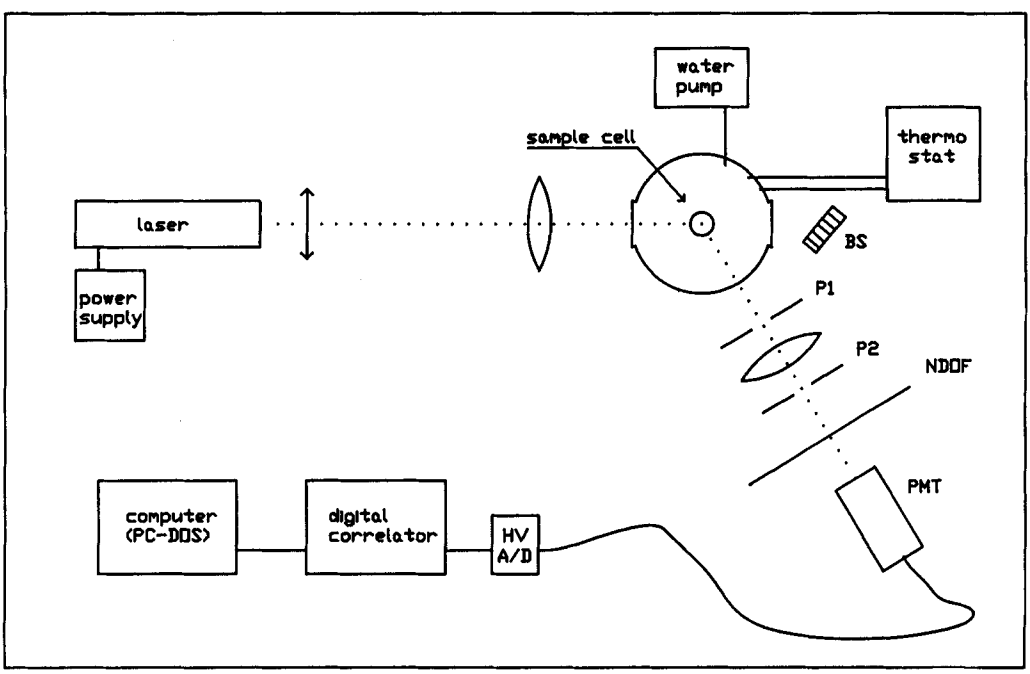

Figure 1 Set-up of photon correlation spectroscopy apparatus. Coherent light is focused by a lens inside a cylindrical glass cell. BS is a beam stop. Pinholes P1 and P2 define the detected area. A narrow band optical filter (NBOF) allows only scattered laser light to register on the photomultiplier (PMT). Pulses are amplified and discriminated (A/D) before entering, as TTL pulses, a 128-channel, 8 bit digital correlator.

$$r = k_B T / 6\pi \eta D$$

which only requires knowledge of k_B (Boltzmann's constant), T (the absolute temperature), and η (the viscosity of the fluid).

The autocorrelograms were deconvoluted to size distribution spectra by full positively constrained Inverse Laplace Transform using the Simplex [12] and the Maximum Entropy algorithm [13] as search and normalizing criteria, respectively. Exhaustive descriptions of the physical foundations and experimental set-ups for PCS experiments are given in the literature [11].

Zeta potentials were measured with a Malvern Zetasizer instrument (Fig. 2). This machine uses a 5 mW He-Ne laser, and it is, correspondingly, less sensitive.

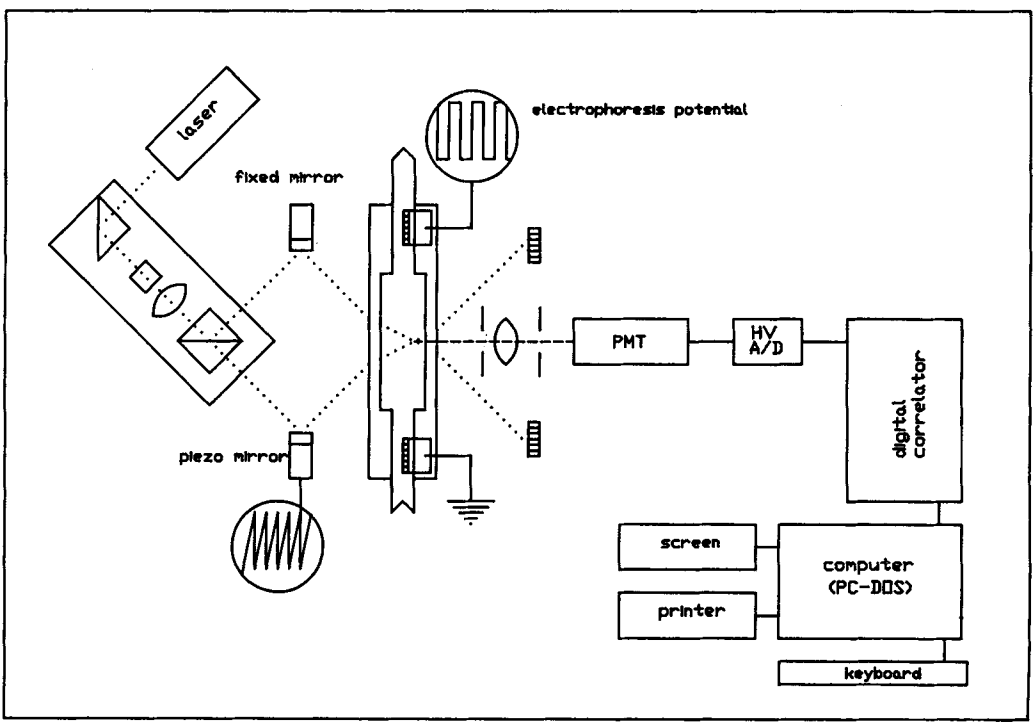

Figure 2 Set-up of the electrophoresis - photon correlation spectroscopy apparatus (Malvern Zetasizer IIc). Light from a He-Ne 5 mW laser is split in two beams, which recombine inside a 5 cm long, 0.4 mm diameter cell at the stationary layer. One of the mirrors vibrates at 200 Hz in order to impose a Doppler shift on the signal. An alternating potential (up to 200 V, 0.5 Hz) is applied through platinized platinum electrodes separated from the sample by semipermeable membranes. Actual potentials are read through sensing electrodes. The area at the crossing ot the two laser beams is observed by a PMT through two pinholes. After amplification and discrimination, pulses are sent to the hardwired digital correlator.

The samples had the following origins: Aldrich Na salt was purchased from Aldrich Chemical Co.; Aldrich purified (hydrogen form) and Gorleben (a groundwater humic

acid) were from TUM, Garching (FRG) and Lake Bradford FSU, Tallahassee (Florida, USA) respectively; Fanay-Augeres (from a granitic groundwater) was purified in our laboratories [14]; podzol, rendzine (from soil) and 'synthetic' humic acid were from Centre de Pedologie Biologique, Nancy (France); sediment came from Laboratoire de Chimie Organique Structurale, Universit Pierre et Marie Curie, Paris (France); Mol (clay water) was from CEN/SCK, Mol (Belgium). 'Synthetic' humic acid was prepared by condensing catechol with triglycine [15].

Preparations were made using freshly ultrafiltered water (Millipore) at a constant temperature in a dark room and in a laminar flow hood in order to prevent dust contamination. Small amounts of NaN_3 were added to stock solutions where appropriate in order to prevent bacterial growth.

Results

The results for eight humic acid samples, one fulvic acid, one groundwater, and a sample of 'synthetic' humic acid are summarized in Table 1, giving the average particle diameter (z-weighted) and scattering intensity, the position of the maximum in the size distribution spectra, and the estimated fraction of humic matter responsible for the scattering signal.

All samples except Mol water were prepared from stock H/FAsolutions obtained by dissolving solid H/FA in excess 0.1 M NaOH. Composition of the samples was: [HA] = 100 mg/l, [$NaClO_4$] = 0.01 M, morpholino-ethane-sulphonic acid [MES] = 0.001 M (as buffer pH = 7.0), with the exception of Fanay fulvic acid (concentration: 1000 mg/l) and Mol water, which were collected, filtered and transported under rigorously anoxic conditions and measured as such. Mol water has a humic acid content of about 200 mg/l, an ionic strength equal to 0.02 M (mainly due to $NaHCO_3$), and a pH of 8.5.

Laser power was 0.5 W, with the exception of the Fanay fulvic acid (1.2 W) and the Mol water (0.15 W) sample. Reported values were normalized to 0.5 W. The observation angle was 90°. Electrolyte scatter (6.3 Kcounts/sec) was subtracted.

Scatterers in the size range 50 - 200 nm were observed in all samples except 'synthetic' humic acid. Fulvic acid contained a significantly lower amount of scatterers (one to two orders of magnitude less), as compared to humic acid from the same source and laboratory. Whether such a small amount of fulvic acid scatterers should be considered significant, or rather as an impurity left from humic acid separation, is open to debate. The existence of these scatterers was confirmed by filtering a solution of purified Aldrich HA (Amicon 15 nm nominal pore size) and observing the retentate by transmission electron microscopy.

Centrifugation in a bench centrifuge (7800 rpm nominal) was found to decrease scattering intensity but to only marginally affect the shape of the computed size distribution, and to have virtually no effect at all on the position of the maximum.

The fraction of humic matter causing the observed scattering, as reported in Table 1, is only a semi-quantitative estimate, computed by comparison with Triton X-100 micelles, on the assumption that refraction index is the same and that Rayleigh scattering

Table 1 Summary of results. Values in parenthesis are interpolated to account for different laser power and/or sample concentration.

	Average Diameter (MZ) / Scattered Intensity (nm) (kcounts s^{-1})				
Sample	non centrifuged	centrifuged 5'	centrifuged 30'	maximum	fraction (%)
Aldrich purified	123 / 492	122 / 495	110 / 495	100	3.9
Aldrich (Na salt)	129 / 533	141 / 711	123 / 556	120	2.5
Lake Bradford	94 / 127	94 / 121	83 / 102	80	2.2
Podzol	246 / 769	191 / 651	156 / 474	150	1.5
Sediment	216 / 469	164 / 367	138 / 295	120	1.4
Rendzine	340 / 172	330 / 116	125 / 58	120	0.3
Fanay HA	808 / 697	433 / 347	203 / 194	100	2.4
Gorleben	123 / 19	132 / 16	72 / 11	60	0.4
Synthetic			555 / 0.4	---	$3*10^{-5}$
	non filtered	centrifuged 5'	filtered 0.45 µm		
Fanay FA	--- / (29)	--- / (18)	--- / (11)	70	(0.03)
Mol Water	250 / (370)		143 / (147)	130	(1.0)

regime applies. A 10 g/l solution of Triton X-100 (9.6 nm diameter micelles) scatters 670 kcounts/sec under identical experimental conditions.

Further investigations on the size and charge of Lake Bradford and purified Aldrich humic acids indicated that:

- pH affects neither scatterers size (Fig. 3) nor zeta potential in the 4 to 9 pH range (samples at pH 8 and 9 contained, as a buffer, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid [HEPES]).
- Fluoride ion, EDTA, surfactants (Triton X-100, SDS) and ultrasound do not affect scatterers size. This indicates that the scatterers are of organic nature.
- Zeta potentials become less negative at higher ionic strength. The potential, within error, is a linear function of $\sqrt{I}$.
- In the presence of increasing amounts of Ca(II) or La(III), the size of scatterers increases abruptly at a definite ion concentration to a larger and still definite value (Fig. 4). Zeta potential, on the other hand, increases smoothly (Fig. 5). Critical concentrations for this Ca(II)- and La(III)-induced flocculation (for 40 µg/l Aldrich purified HA, 0.1 M $NaClO_4$, 0.001 M MES, pH 7) are about $5x10^{-3}$ and $7x10^{-5}$M, respectively, which is in good agreement with the Schulze-Hardy rule [16].

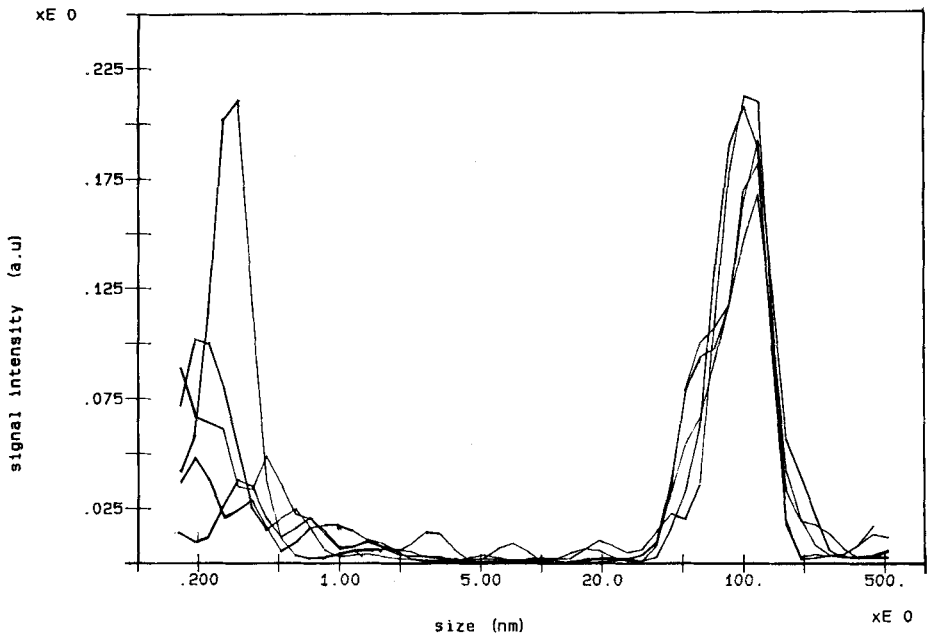

Figure 3 Size spectra of humic acid solutions. Lake Bradford HA, 42 mg/l, in 0.1 M $NaClO_4$, $1x10^{-4}$ M buffer (MES pH 4 to 7, HEPES pH 8 and 9). Signal around 0.3 nm is due to water.

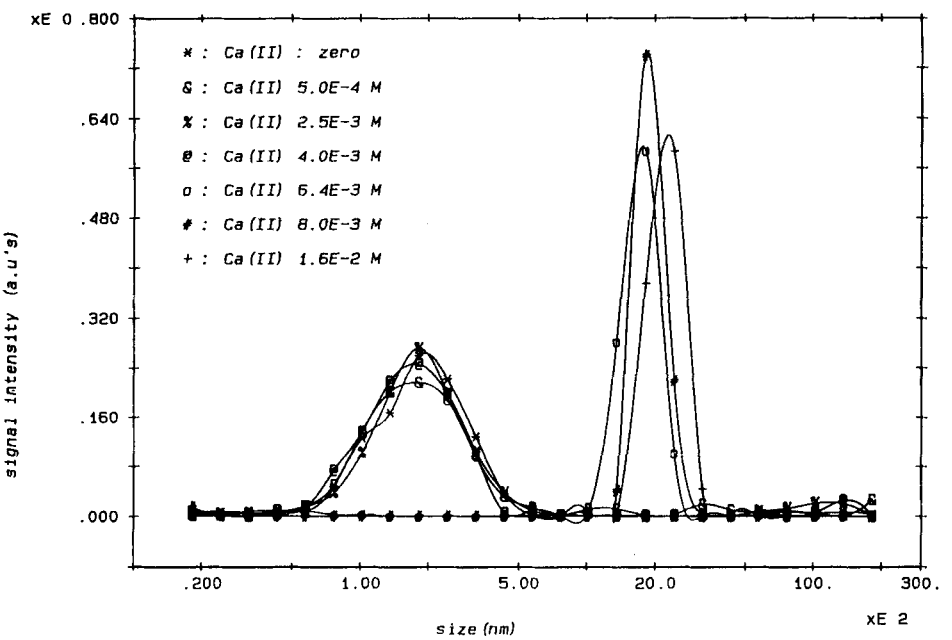

Figure 4 Intensity *vs* size distribution of scatterers in humic acid sample as a function of Ca(II) concentration. Aldrich purified HA, 40 mg/l, in 0.1 M $NaClO_4$, 0.001 M MES, pH 7.

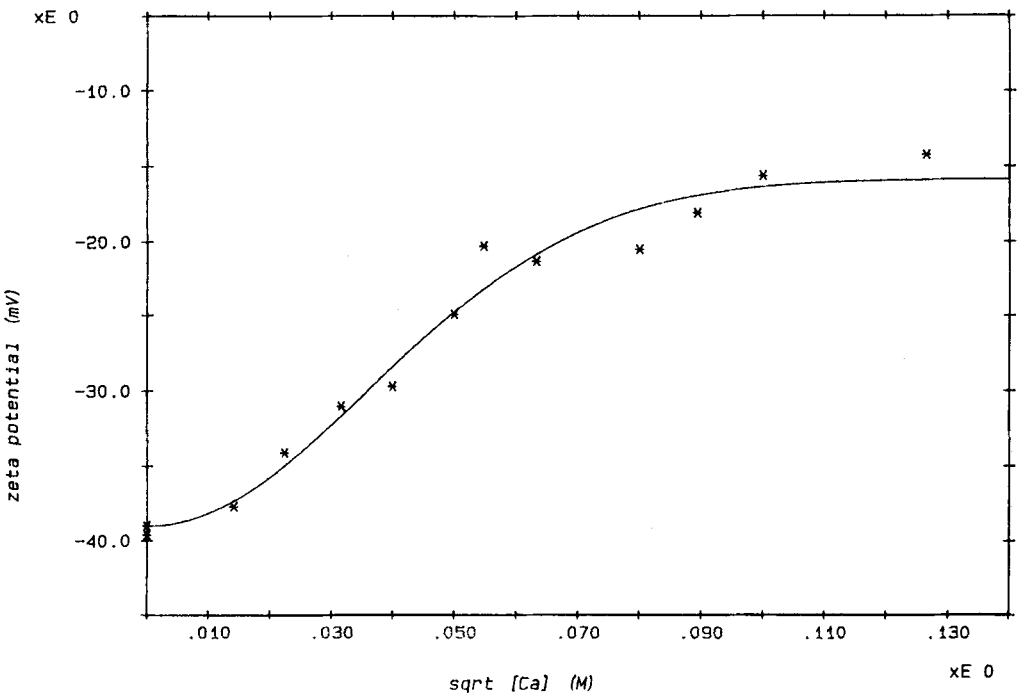

Figure 5 Zeta potential of scatterers in humic acid sample as a function of square root of Ca(II) concentration. Samples as in Fig. 4. Solid line is best least-square fit to equation Z = A - (-39.3 - A) exp(-C[Ca(II)]) (mV), with A = -15.9 ± 1.4 mV, and C = 395 ± 70 M^{-1}.

Conclusions

Large (60-200 nm), negatively charged, organic particles constitute a small (less than 4% in weight) but definite fraction of humic substances. Their existence may explain some results obtained by size-exclusion chromatography. Their possible role in the retention and mobilization of organics and metals in the environment, as catalysts for the hydrolysis of organics, and as nucleation centers for mineral precipitation, is open to discussion.

Acknowledgements

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The Behaviour of Diborane-Reduced Fulvic Acids in Flash Pyrolysis

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Abstract

It is suggested that the striking structural changes introduced in humic substances by reduction with diborane may be useful in studying the role of carboxyl groups in the behaviour of these substances in flash pyrolysis. In the preliminary results shown in this communication, pyrograms of two fulvic acids of different origins and the corresponding diborane reduced substances are compared. It was found that the diborane reduction was responsible for both qualitative and quantitative changes in the pyrolytic patterns of the fulvic acids. These patterns reflect the changes in the reactivity and structural stability of the fulvic materials caused by the disappearance of the carboxyl groups.

Introduction

The progress in the application of analytical pyrolysis (Py) to the study of humic substances (HS) has been limited not only by technical factors [1], but mainly by the intrinsic complex chemical nature of these materials. This complexity has lead to serious difficulties in the interpretation of their complex pyrolytic patterns.

Recent reviews of the extense bibliography on this topic [2-5] show that Py, in combination with gas chromatography and mass spectrometry (Py-GC-MS, Py-MS), can be used as fingerprint techniques, illustrating the differences between humic fractions of various origin [6,7]. However, in contrast with the well known pathways of thermal breakdown of synthetic polymers [8], the mechanisms involved during the pyrolysis of HS are not completely understood.

The main structural information concerning the parent macromolecules obtained by Py is related to the identification of different components of the humic extracts. In fact, many typical Py-products of HS were recognized by comparison with those obtained by Py-GC-MS of well defined biopolymers [9-13].

Some approaches to the study of the Py mechanisms of HS have been carried out by examining the influence of chemical alterations of the humic molecules [14,15]. The specific modification of carboxyl groups (COOH), usually based on the preparation of ester derivatives, has not always been satisfactorily accomplished. In general, the esterification conditions are either too drastic (when methanol-HCl is used) or suffer from poor yields and non-specificity, when diazomethane is employed.

In a previous study [16] we were able to obtain stable FA preparations in the reduced state upon treatment with diborane in tetrahydrofuran (THF). The diborane treatment was found to be a useful method to obtain chemically transformed HS without carboxyl groups. This striking alteration is responsible for changes not only in the solubility and colloidal properties of this transformed HS, but probably also in the nature of the different intramolecular forces affecting the structural arrangement of the macromolecular constituents. Therefore, it is expected that the comparison by Py-GC-MS of original and reduced humic samples can provide information on the effects of carboxyl functionality upon the behaviour of HS against thermal degradation.

Materials and Methods

Two fulvic acids isolated from a podzol soil (FA-P) and from the lake water over a peatland (FA-W) were investigated. The processes for extraction and purification, as well as the physico-chemical characteristics of these samples have been reported elsewhere [17,18].

The treatment of the samples with diborane in THF to obtain the reduced preparations has been previously described [16]. Briefly, the samples were dissolved in a minimum amount of THF, and 5 ml of a 1M solution of diborane in THF (Aldrich) was slowly added under N_2 atmosphere at a temperature of 0°C. Additional amounts of diborane in THF were added periodically until hydrogen evolution was completed; the flask was stoppered under N_2 and heated to 50°C. After 20 days, the excess of diborane was destroyed by the addition of water; the tetrahydrofuran was evaporated under reduced pressure, and the boric acid formed was removed by the successive evaporation in the presence of methanol. The efficiency of the reaction was confirmed by the more or less complete disappearance of the carbonyl stretching absorption at 1720 cm^{-1} in the IR spectra, and the disappearance of the resonances for carboxyl carbons at about 175 ppm, in the ^{13}C NMR spectra.

Pyrolysis Conditions

The pyrolysis was carried out at 700°C in a CDS Pyroprobe 190 consisting of a Pt coil heated by an electric current at a rate of up to 20°C/$msec^{-1}$. The sample (2 mg) was placed in a quartz tube, using quartz wool for end plugs. The pyrolysis unit was mounted in the injection block of a HP 5730A gas chromatograph, or on a HP 5992B in the case of the Py-GC-MS system, through the resistively heated special device shown in Fig. 1, which allows the horizontal insertion of the pyrolyzer.

The volatile Py products were separated on a 25 m cross-linked fused silica column (i.d.=0.32 mm) coated with OV-101 (0.11 μm film thickness). Before starting the pyrolysis temperature program, a pre-heating of the pyrolysis unit at 250°C for 10 min allowed adhered lipid material to be removed, channeling any evolved material directly to the purge vent. The Py products were first concentrated in a loop of the column into a liquid N_2 cold trap and then the GC oven was heated from 40 to 300°C at a rate of 6°C/min. The Py products were identified by comparing their EI mass spectra with mass spectra libraries, and with mass spectra and GC retention times of standard compounds.

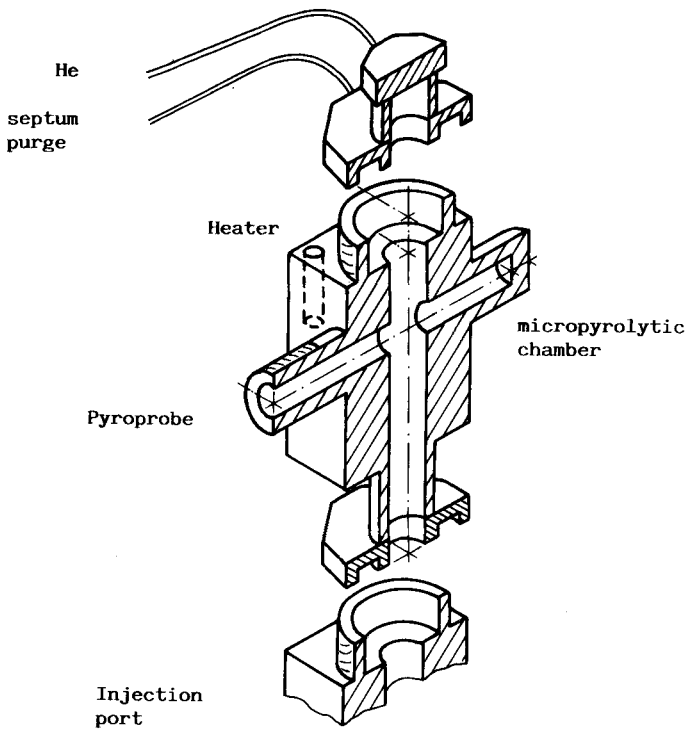

Figure 1 Expanded view of the pyrolytic accessory.

Results and Discussion

The great qualitative and quantitative differences between the pyrolytic patterns yielded by the original and diborane-reduced samples are evident (Figs 2 and 3). The original samples gave relatively simple pyrograms, as is usual for these humic fractions [17], whereas the reduced samples yielded complex chromatograms. With some exceptions identification of the Py compounds in the pyrograms was achieved (Table 1). There are many Py products, especially in the reduced samples, and Table 1 shows only the main series of compounds present, before and after diborane reduction. This exclusion is possible, since a detailed description of the identified compounds is not required in order to characterize the changes occurring upon reduction. Only significative peaks are labelled in the Figs 2 and 3 to illustrate the different nature of the Py products.

The Py products encountered in the original samples were very similar to those previously described [5, and references therein]. The podzol FA (FA-P), as other soil FA's, yielded mainly Py products from polysaccharides, together with some aromatic compounds from lignins and dialkyl phthalates. No nitrogen derivatives were identified. The FA-W gave a similar pattern of Py products, with a higher proportion of aromatic compounds, whereas dialkyl phthalates were not frequently encountered.

Table 1 Series of Py products identified in the original and reduced samples.

	FA-P	FA-P-R	FA-W	FA-W-R
Low b.p. compounds	+	+++	+	+++
n-alkanes (C8-C33)	+	++	+	++
Branched hydrocarbons	-	++	-	+
Furan,furfural,benzo-furan derivatives	+	+	+	+
Aromatic compounds from lignins	+	+	+	+
Hydroaromatics	-	++	-	++
Alkyl benzenes	-	+	+	+++
Alkyl naphthalenes	-	++	-	+

- not detected
+,++,+++ : low, medium and high presence

The reduced preparations (FA-P-R and FA-W-R) gave different types and amounts of Py products. In both cases the predominant components were series of multibranched aliphatic and aromatic hydrocarbons (labelled a and b in the chromatograms); the former were predominant in FA-P-R and the latter in the FA-W-R.

It is suggested that the loss of functionality leads to "more easily pyrolyzable samples", and these samples with a lower oxygen content yield higher relative amounts of Py products. These data are in agreement with the previous observations of Bracewell *et al.* [19] on the transformations occurring during the Py of organic matter from surface organic horizons. They found that, upon Py, raw humus originating from a retarded humification process involving the selective removal of functional groups containing oxygen and nitrogen, yielded high amounts of hydrocarbons. Py should also cause aromatic moieties from lignins and amino acids to lose functional groups and thereby yield aromatic hydrocarbons. However, aromatic hydrocarbons can also arise, by cyclisation of highly unsaturated chains, which can be formed during Py by elimination of electron-withdrawing side groups, such as hydroxyl and carboxyl groups [20].

Another explanation could be that the reductive treatment has progressed to complete the conversion of COOH into CH_3 groups. This would mean that after pyrolysis there would be more possibilities for alkyl rearrangements and reactions among alkyl radicals in the reduced samples. In fact, when appropriate electron-donating groups are present, complete reduction of COOH to a methyl group is possible [21]. Thus, studies of the diborane reduction of indole and pyrrole carbonyl derivatives, and of other "electron-rich" aromatic carbonyl compounds, showed that the carbonyl group was completely reduced to a methylene group [22].

From the studied samples it was not possible to select the most significant compounds to monitor the particular behaviour of some humus components. In general, all the

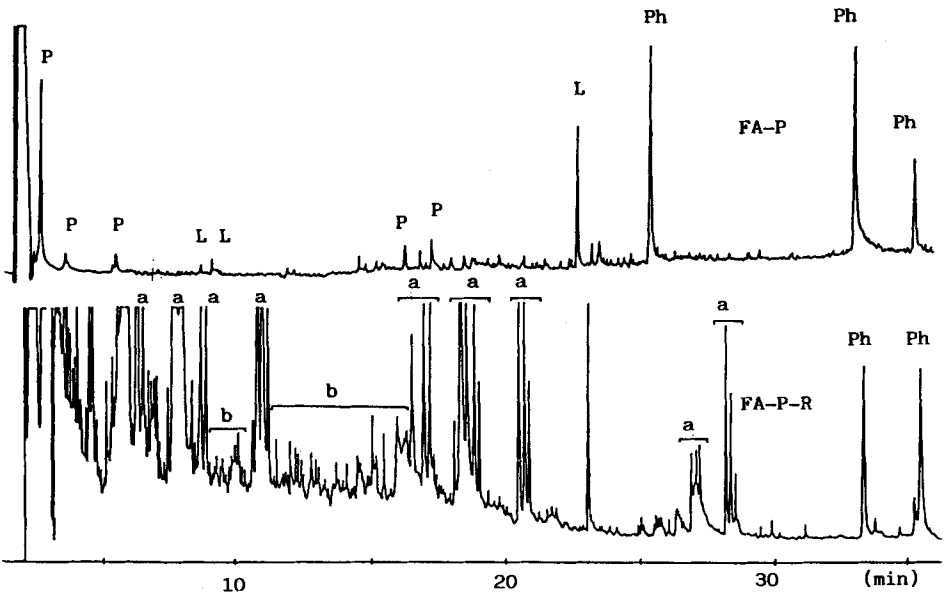

Figure 2 Pyrograms of the Podzol fulvic acid before (FA-P) and after (FA-P-R) the reductive treatment. Py-products characteristic for polysaccharides (P), lignin (L), dialkyl phthalates (Ph), multibranched aliphatic (a) and aromatic (b) hydrocarbons are indicated.

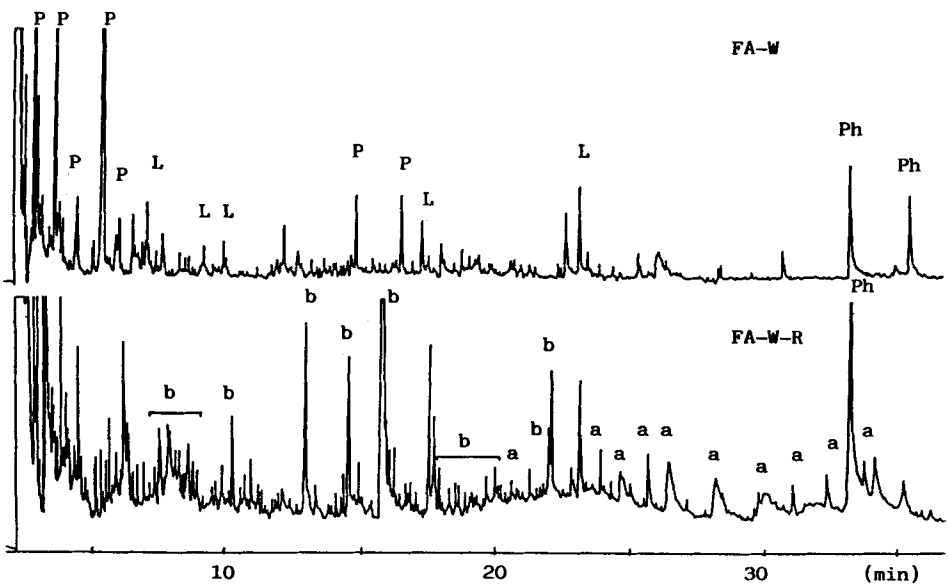

Figure 3 Pyrograms of the peat water fulvic acid before (FA-W) and after (FA-W-R) the reductive treatment. Labelled compounds as in Fig. 2.

identified compounds in the original samples were also present in the pyrograms of the reduced samples. Therefore, the diborane treatment does not apparently affect the polysaccharide moieties responsible for most of the Py products of the original samples. The present results should, however, be corroborated by further investigations with model compounds. Until now it has been demonstrated that the application of the hydroboration reaction to carbohydrates containing either a terminal or an endocyclic double bond yielded only hydration products [23].

The evolution of nitrogen compounds arising from Py of polypeptides would be very interesting, since several authors have shown that the treatment with diborane can be adapted for the specific reduction of carboxyl groups in amino acids, peptides and proteins [24, 25] without affecting the peptide bonds. Investigations on the particular behaviour of nitrogen-containing Py products are currently in progress.

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Some Aspects of the Characterization of Humic Substances in Lake Waters

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Abstract

Several different parameters, including elemental and functional group analyses, solid state ^{13}C NMR spectroscopy and E4/E6 ratios, were measured on the dissolved organic matter isolated and fractionated with ultrafiltration and XAD-8 techniques from two highly colored lakes in Finland. The results of the analyses are discussed in relation to the sample isolation (DOM, FA or HA). Multivariate analyses showed some trends in the average molecular masses, in the results of elemental analyses and, especially, in the characteristics of the ^{13}C NMR spectra so far obtained.

Introduction

In another paper [1] we described a systematic study on the isolation and fractionation of aquatic humus. The aim of the present report was to find common characteristics for the fractions isolated in the previous study by using UV-determination of E_4/E_6 ratios, elemental and functional group analyses, determination of average molecular masses (Mw, weight average), ^{13}C NMR, and multivariate analyses.

Materials and Methods

The DOM-fractions studied originate from the work presented in the paper by Peuravuori and Pihlaja [1]. Elemental analyses (C, H, N) of the freeze-dried materials were performed by various combustion techniques on a Carlo Erba Elemental Analyzer at the University of Joensuu. The concentrations of the acidic functional groups of the freeze-dried materials were estimated by titrating with NaOH [2]. The solid state ^{13}C NMR spectra (CP/MAS technique) of the freeze-dried materials were measured on a Bruker MSL-100 instrument at 25.178 MHz at the University of

Umeå. Experimental conditions: 1 ms contact time, 2.5 s repetition rate, 700 data points zero filled to 2K and 3.5 KHz spinning speed using the double air-bearing probe and Al_2O_3 rotors. The chemical shift scale is referenced to adamantine at 964 Hz (δCH_2 = 38.3 ppm). The E_4/E_6 ratios were measured conventionally [3] at 465 and 665 nm, respectively.

Results

Table 1a lists the amounts and distributions of the fractions isolated from the water samples from lakes Savojärvi (S) and Mekkojärvi (M1 and M2) [1].

Table 1a Amounts and distributions of the different ultrafiltration fractions of the water samples S (taken on February 15, 1988), M1 (taken on May 16, 1988) and M2 (taken on September 30, 1988) together with amounts of humic (HA) and fulvic acids (FA) and neutral substances (MeOH) separated with the XAD-8 technique (d-% = decrease-%).

Savojärvi (S)				Mekkojärvi (M1)				Mekkojärvi (M2)			
	mg/l	d-%	UF-%		mg/l	d-%	UF-%		mg/l	d-%	UF-%
UF.I		(nominal molecular mass cutoff of UF-membrane >10^5)									
(a)	4.4		7.9	(a)	1.3		2.9	(a)	6.9		9.6
(b)	4.2	4.5		(b)	1.2	7.7		(b)	6.6	4.3	
(c)	3.1	29.5		(c)	1.0	23.0		(c)	5.5	21.0	
UF.II		(nominal molecular mass cutoff of UF-membrane 10^4-10^5)									
(a)	10.7		19.3	(a)	15.2		34.0	(a)	25.2		34.8
(b)	8.7	18.7		(b)	12.8	16.0		(b)	22.1	12.2	
(c)	7.7	28.0		(c)	11.4	24.9		(c)	19.5	22.6	
UF.III		(nominal molecular mass cutoff of UF-membrane 10^3-10^4)									
(a)	40.3		72.8	(a)	28.1		63.1	(a)	40.4		55.7
(b)	22.7	43.7		(b)	22.1	21.5		(b)	32.9	18.6	
(c)	17.8	55.8		(c)	13.7	51.5		(c)	21.8	45.9	
UF.IV		(nominal molecular mass cutoff of UF-membrane <10^3)									
(c)	0.72			(c)	2.2			(c)	2.1		

Table 1b Amounts of the hydrophobic humic (HA+FA) and neutral (MeOH) substances separated with the XAD-8 technique from the minor subsamples.

	mg/l	HA/FA %		mg/l	HA/FA %		mg/l	HA/FA %
HA	4.3	17.8	HA	4.3	18.2	HA	7.7	21.2
FA	19.8	82.2	FA	19.3	81.8	FA	28.7	78.8
HA+FA	24.1		HA+FA	23.6		HA+FA	36.4	
(MeOH)	1.6		(MeOH)	1.1		(MeOH)	4.6	

Table 1b shows the amounts of humic and fulvic acids and neutral hydrophobic substances (MeOH) separated from the minor subsamples of S, M1, and M2 with the

XAD-8 technique [1]. The symbols a-c in Table 1 correspond to untreated, freeze-dried UF-concentrates (a); the same after a cation-exchange (b); or after XAD-8 treatment followed by cation-exchange (c) [1]. Experimental data for several parameters are collected in Table 2. The results of different multivariate analyses [4] based on the collected data are shown in Fig. 1-3 and discussed below.

Table 2 Results of different analyses for the water samples S, M1 and M2. The ^{13}C-ranges used were: aliphatics 0-50, carbohydrates 50-110 and aromatics 110-160 ppm.

Fraction	Mw	H/C	O/C	N/C	E_4/E_6	$COOH_{tot}$	Ar-OH	Aliph.	Carboh.	Arom.
		atomic ratio				meq/g	meq/g	%	%	%
SUF.Ia	150000	1.237	0.985	0.022	7.078	4.73	0.68	51.1	34.0	14.9
SUF.Ib	126900	1.424	1.182	0.026	7.250	4.65	0.97	46.6	41.5	11.8
SUF.Ic	43900	1.149	0.931	0.018	6.991	4.78	0.42	49.4	33.0	17.6
SUF.IIa	74400	1.283	1.092	0.018	8.868	4.32	0.97	45.8	41.5	12.7
SUF.IIb	21400	1.040	0.791	0.015	7.084	4.44	0.44	35.0	45.0	20.0
SUF.IIc	20900	1.018	0.789	0.015	6.630	4.02	0.40	37.2	40.4	22.4
SUF.IIIa	5400	1.546	1.743	0.020	9.725	4.73	0.76	44.9	32.4	22.7
SUF.IIIb	5700	1.420	1.425	0.014	9.787	4.86	0.48	33.7	37.9	28.5
SUF.IIIc	7000	1.070	0.751	0.012	9.186	5.16	0.42	34.9	36.4	28.7
SUF.IVc	3500	1.231	0.573	0.014	10.800	5.45	0.79	61.3	23.9	14.7
SHA	26200	0.992	0.719	0.019	6.097	4.56	1.44	35.2	36.2	28.7
SFA	8400	1.051	0.786	0.009	7.715	4.75	1.36	36.4	37.1	26.4
SMeOH	6400	1.245	0.746	0.012	10.833	0.72	0.01	52.4	30.0	17.6
M1UF.Ia	82700	1.389	0.876	0.038	7.213	3.85	0.87	41.9	46.8	11.3
M1UF.Ib	46400	1.270	0.763	0.032	7.170	4.33	0.74	39.7	44.8	15.5
M1UF.Ic	28500	1.172	0.918	0.032	6.468	4.93	0.53	42.8	40.1	17.1
M1UF.IIa	33000	1.155	0.983	0.017	8.542	4.84	0.98	43.9	40.4	15.7
M1UF.IIb	23200	1.007	0.819	0.018	6.018	4.44	0.72	36.1	40.4	23.5
M1UF.IIc	25000	0.983	0.776	0.019	8.396	5.01	0.37	34.9	40.3	24.7
M1UF.IIIa	7200	1.530	1.826	0.021	10.679	4.57	0.57	45.3	36.5	18.2
M1UF.IIIb	7200	1.473	1.552	0.018	9.976	5.11	0.40	34.6	38.1	27.4
M1UF.IIIc	7400	1.057	0.805	0.014	9.206	5.24	0.44	37.5	34.9	27.6
M1UF.IVc	5100	1.169	0.565	0.011	9.619	4.40	0.61	59.3	26.4	14.3
M1HA	25500	0.940	0.690	0.023	6.933	4.65	1.42	37.0	33.6	29.4
M1FA	11300	0.990	0.716	0.013	7.538	5.20	1.22	36.8	35.7	27.6
M1MeOH	6600	1.174	0.696	0.014	10.350	0.61	0.01	55.3	31.0	13.7
M2UF.Ia	102000	1.149	0.890	0.012	7.705	4.21	0.67			
M2UF.Ib	69500	1.026	0.696	0.014	6.857	3.81	0.40			
M2UF.Ic	67200	1.048	0.736	0.018	6.925	4.00	0.53			
M2UF.IIa	44400	1.137	0.964	0.016	9.167	4.37	0.88			
M2UF.IIb	27300	0.950	0.746	0.013	8.025	3.82	0.71			
M2UF.IIc	25900	0.951	0.747	0.015	7.652	4.59	0.35			
M2UF.IIIa	9700	1.236	1.278	0.012	10.833	4.66	0.85			
M2UF.IIIb	8900	1.072	0.933	0.011	9.556	5.88	0.42			
M2UF.IIIc	10000	0.937	0.740	0.010	9.333	4.29	0.39			
M2UF.IVc	6200	1.150	0.568	0.007	7.372	4.99	0.71			
M2HA	29400	0.935	0.649	0.018	7.690	4.52	1.22			
M2FA	12500	0.979	0.664	0.011	9.778	5.53	1.02			
M2MeOH	9000	1.877	0.667	0.009	9.867	0.50	0.01			
NoHA	26800	0.868	0.658	0.024	9.580	4.57	1.46	31.3	37.1	31.6
NoFA	8100	0.930	0.690	0.016	9.146	5.36	1.38	33.5	38.5	28.0

NoHA and NoFA are Nordic Reference Standards (humic and fulvic acid, respectively).

Discussion

E_4/E_6 Ratio

The correlation of the E_4/E_6 ratio (Table 2) with the average molecular masses (Mw, weight average) of the different fractions was not very good (r=-0.57, n=20), but nevertheless agrees with the postulation that the ratio should decrease with the increasing molecular size [3]. Even poorer correlations were found with the H/C ratio and aromaticity (Table 2), although the E_4/E_6 ratio appears roughly to increase with the increasing H/C ratio [5]. As a whole our results support the view that the E_4/E_6 ratio has no direct relationship to the nature of the water soluble humic substances nor to the content of condensed aromatic rings in HA and FA [3].

Functional Group Analyses

The titrimetric total concentration of the COOH-groups and that of the Ar-OH correlated reasonably well ($r \approx 0.7$) with the relative carbon contents (solid state NMR: 160-190 and 140-160 ppm, respectively). No other significant correlations were found, although the titrimetric contents of the functional groups (Table 2) are in accordance with those described in the literature [6]. However, our results did not allow a clear distinction between the FA- and HA-fractions.

Elemental Analyses

The H/C, O/C, and N/C ratios based on the average of three parallel C,H,N-analyses (the content of oxygen was taken as a difference from 100 %) are shown in Table 2. The H/C, O/C and N/C ratios are similar to the values of aquatic humic substances reported earlier [7]. Of the UF-concentrates, those obtained by method (c) gave the smallest H/C ratios, especially in the case of the UF.III-concentrates (Fig. 1). The O/C ratio behaved similarly but only in the UF.III category. This is not at all reflected by the carboxylic group contents and only moderately by the Ar-OH contents (Table II). The ability of the XAD-8 treatment to remove carbohydrates may, however, partly explain the decrease in the O/C ratio when applying method c. The N/C ratios alone do not allow any specific discussion.

The peculiar behavior of the UF.I-concentrates (Fig. 1) supports the postulation [8] that ultrafiltration can be subject to interactions which increase values of molecular masses, whereas pH-adjustment (method c) can result in lower molecular masses by disrupting interactions between the humic material and e.g. Fe and Al. Fig. 1d shows the H/C vs O/C Van Krevelen plot for all UF-fractions obtained by method (c) together with the HA- and FA-fractions isolated from the original water samples with the XAD-8-technique. It is also indicated that UF.IC-concentrates (cutoff $>10^5$) as a whole were not alike the other (c)-concentrates. Furthermore fractions UF.IVc (cutoff $<10^3$) formed another distinct cluster with high H/C values, which speaks for increased aromaticity [9]. All other fractions were practically clustered together with the humic substances (HA and FA). Here the HA-materials are always placed to the left of and below the FA homologues as suggested earlier

[9]. The M1 and M2 fractions do not fall closely together in plot 1d, which is obviously an indication of the difference between the spring and autumn sample.

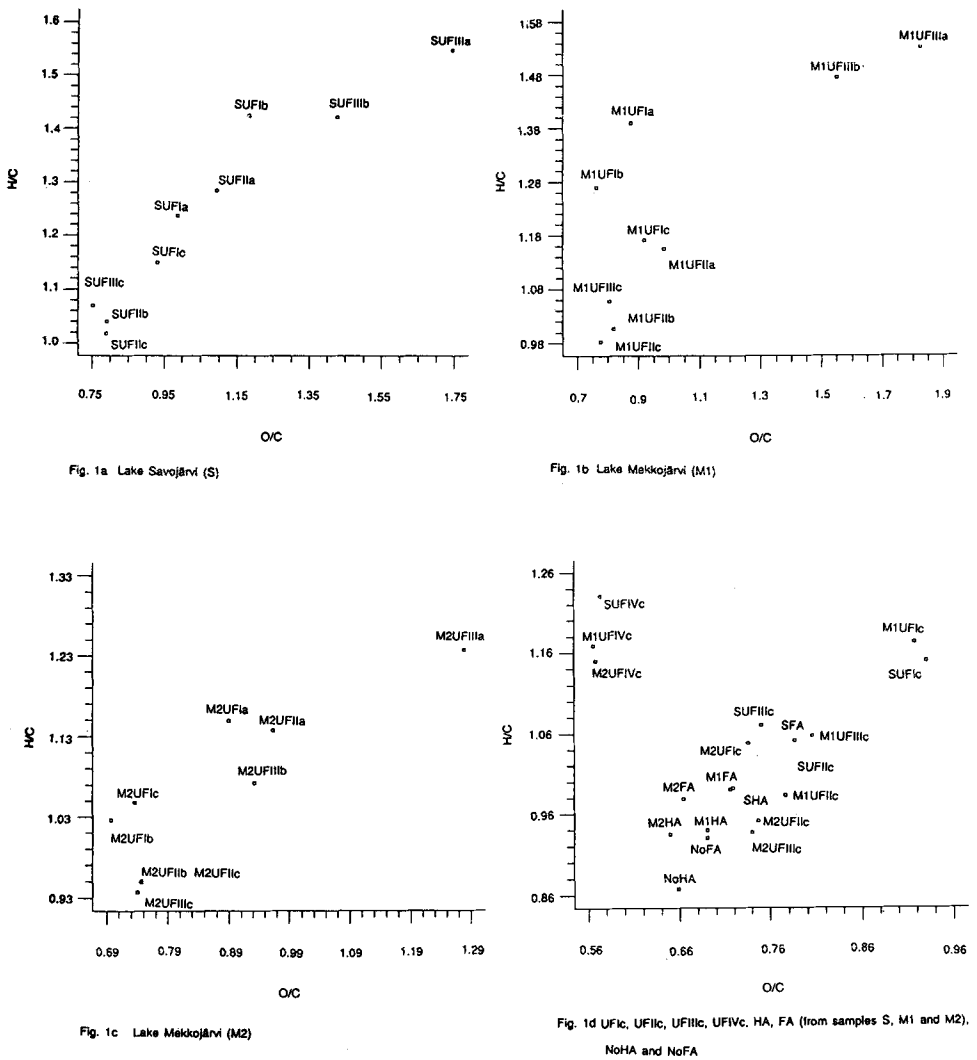

Fig. 1a Lake Savojärvi (S)

Fig. 1b Lake Mekkojärvi (M1)

Fig. 1c Lake Mekkojärvi (M2)

Fig. 1d UFIc, UFIIc, UFIIIc, UFIVc, HA, FA (from samples S, M1 and M2), NoHA and NoFA

Figure 1 Van Krevelen plots for several groups of samples from the present study.

^{13}C NMR

Typical examples of the ^{13}C NMR spectra are shown in Fig. 2. According to the spectra the aromatic fraction between 110 and 140 ppm increased when carrying out the cation exchange (method b) on the original type a UF-concentrates. Method b

decreased the relative amount of the aliphatic fraction (0-50 ppm) more than method c, especially in the cutoff ranges 10^4-10^5 and 10^3-10^4. Despite the fact that 97% of the carbon atoms present have been stated to appear in the solid state NMR spectra [10], it is not possible by inspection of the spectra alone to draw similar conclusions as, e.g., from Fig. 1d. However, certain deductions about the structural variations can be made quite easily on the basis of the percentage constitutions given in Table 2 [11].

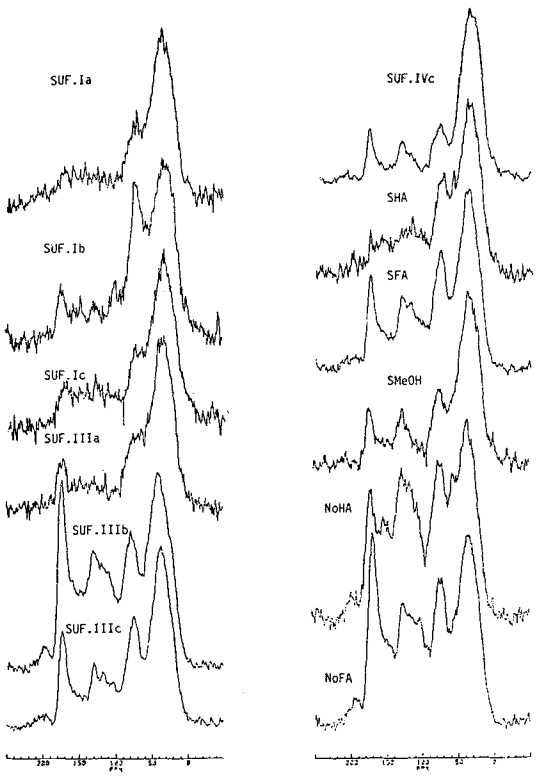

Figure 2 Typical examples of the ^{13}C CP/MAS NMR spectra.

Multivariate Analysis

It was not possible to find any single parameter specific for a certain group of fractions or to HA and FA. A multivariate analysis based on average molecular mass (Mw or Mn), H/C, O/C, N/C, aliphatic, aromatic, and carbohydrate contents, as well as on aromatic/aliphatic and aromatic/carbohydrate ratios, was proved to be the best approach. Principal component analysis (PCA) is a useful technique for reducing the number of variables in a data set by finding linear combinations of those variables that explain most of the variability [4]. In addition to the E_4/E_6 ratios [12] the functional group contents cannot be applied unambiguously to resolve different sample categories. Fig. 3 shows two example plots against the first two principal

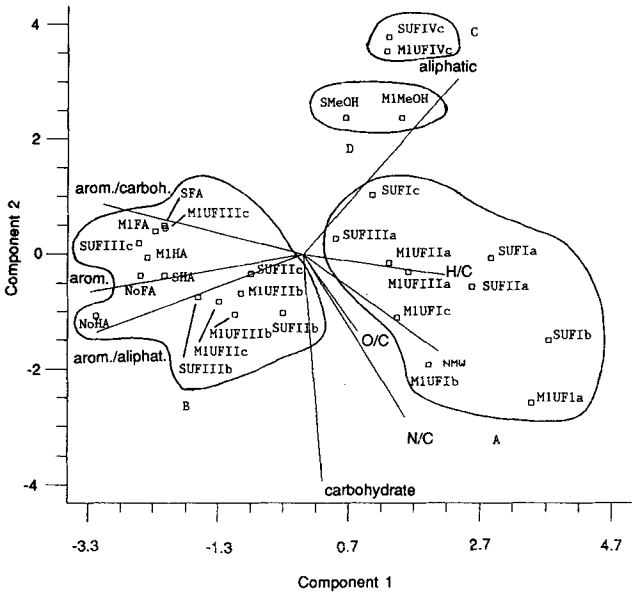

Fig. 3a Biplot of first two principal components

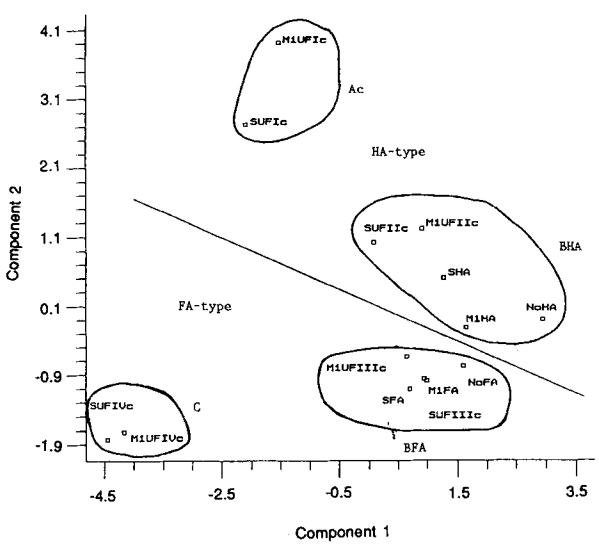

Fig. 3b Scatterplot of first two principal components

Figure 3 PCA plots for the materials studied.

components of the above variables. Since the NMR data were not yet available for the M2 material, the analysis consisted only of the data for samples S and M1.

In the biplot (Fig. 3a) the first component accounts for 46.4% of the total variability and the second component increased the statement level to a fair 70.9%. Four clearly separated clusters (A-D) can be seen in Fig. 3. Cluster A contains all UF.I-concentrates (types a, b and c) together with all other original UF-concentrates (only type a). All of the other UF-concentrates together with the HA-and FA-substances from the original water samples and with the Nordic Reference Standards (NoHA and NoFA; Table 2), fell into cluster B. As expected, two minor clusters were formed by materials of the nominal molecular mass cutoff $<10^3$ (C, Tables 1 and 2) and MeOH-extracts (D, Tables 1 and 2). Cluster C can also be seen in Fig. 3b (levels of statement 53.3 and 82.1% for the first and second PC, respectively), which actually demonstrates the clustering of HA- and FA-type substances (BHA and BFA, respectively). It also shows that clusters Ac and C, containing the UF.I-concentrates and UF.IV-filtrates, respectively, treated by method c are very well separated from clusters BHA and BFA.

To conclude, it can be stated that the cutoff range 10^3-10^4 in the ultrafiltration (UF.III-concentrates) is quantitatively largest, and the separated materials closest resemble fulvic acids.

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